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ERRATUM

K. V. Jubb, L. Z. Saunders and Helen V. Coates, Vol. 67, No. 1, Page 29 Legends to Figs. 1–11 should read ×480, ×200, ×360, ×1,000, ×1,000, ×1,000, ×1,000, ×150, ×30, ×35, and ×21, respectively.

THE DEVELOPMENT OF MALE GENITALIA IN AN ABNORMAL CALF FOETUS

By

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INTRODUCTION

The numerous factors controlling development of any body system are intricately inter-related. It is therefore difficult to separate them and estimate their relative importance. In the case of the genitalia the role of the sex hormones in development is usually emphasised and undoubtedly their action is largely specific to that system. In an analysis of factors influencing the development of the reproductive tract of several species Price (1947) concluded that the testes, prostate, and seminal vesicles respond to prenatal hormonal stimulation. Jost (1947) showed that castration of foetal rabbits retarded growth of the male reproductive tract. Wells and Fralick (1951) concluded that the foetal rat testis produces a hormone affecting growth of the male genital organs. The condition of free-martinism (Lillie, 1917) in cattle indicates the importance of hormonal factors in prenatal development of the reproductive tract in that species. Although it is less easy to estimate the importance of other factors, it is unwise to ignore them.

The general development of the structures topographically related to the reproductive tract but not specifically controlled by sex hormones could affect the development of this sytem. An animal in which the surrounding structure is abnormal but hormonal control normal might provide indications of the importance of general body structure in normal development of that organ system. Such an animal, perhaps, is the Schistosomus reflexus type of monstrosity. Williams (1943, 1947) describes this monstrosity as resulting from a dorsal instead of a ventral reflection of the somatopleure of the blastoderm. This results in the peritoneum and pleura forming the external covering instead of skin. The thoracic and abdominal viscera lie free in the allantoic cavity and the spinal column is folded dorsally, with the limbs and head more or less completely enclosed within the body walls.

Through the courtesy of the Department of Veterinary Surgery we were able to examine a full term male specimen of this type of monstrosity. To establish the stage of development of various regions of the Schistosomus reflexus a series of nine normal bovine foetuses of different sizes were also examined.

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OBSERVATIONS

Schistosomus reflexus

The external genitalia. The structures to be described were found on the skin stretching from the root of the tail to what would normally be the umbilicus and include the opening of the alimentary

tract, the scrotum, the opening of the sheath, the penis and the teats. The first three structures were in line with each other.

The term 'opening of alimentary tract' is used as, although it was found immediately under the root of the tail, it resembled more a simple turning in of perineal skin than an anus. The opening was 50 mm. from the middle of the scrotum which was 18 mm. in height. When sectioned the latter was found to be solid and to consist of skin and subcutaneous tissue. There were no testicles or associated structures. Externally it appeared to be a normal scrotal sac prior to the descent of the gonads, but was furnished with hair.

The orifice of the sheath was normal in external appearance, 150 mm. from the middle of the scrotum and 50 mm. from the umbilicus. On opening the sheath it could be seen that the penis lay in a normal position. The pre-penile portion of the sheath was 35 mm. in length and the 'free' part of the penis 25 mm. The penis was adherent to the prepuce and the frenulum was persistent. On further dissection the sigmoid flexure was found in a normal position relative to the scrotum. The measurement from tip of the penis to the sigmoid flexure at the insertion of the retractor penis muscle was 75 mm., and from this point to the distal extremity of the bulbo-cavernosus muscle was 50 mm. At the level of the bulbo-cavernosus muscle the penis measured 6 mm. x 8 mm. in cross-section and at the proximal extremity of the free end 4 mm. x 6 mm. Four rudimentary teats were present ; a pair on either side of the mid line. Their position is shown in Fig. 1.

Fig. 1.

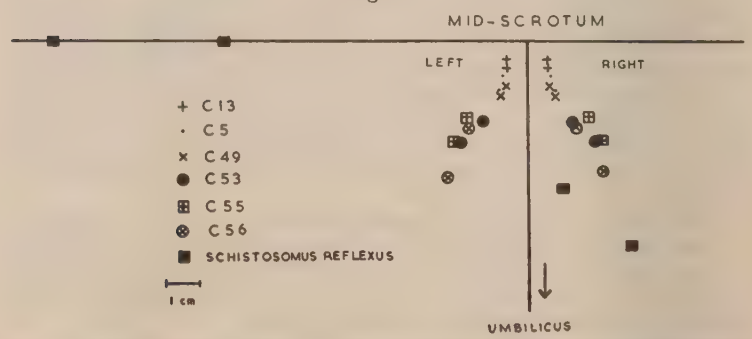


Fig. 1. Diagram showing positions of teats in six normal foetuses and in the Schistosomus reflexus. (The positions of the teats in the three other normal foetuses (C4, C9, C54) also agree with the normal arrangement but are omitted for clarity.)

The internal genitalia. The testicles were caudal to the kidneys at the level of the ilia, the left being rather more cranial and slightly larger than the right. Although the testicles appeared macroscopically normal each epididymis was abnormal in arrangement.

It was attached only to one surface of the testicle, the caput being markedly reduced. The vas deferens was not reflected on to the epididymis in the region of the caudal pole of the testis, but immediately left the organ as a convoluted tube gradually thinning out to a normal simple tube which thickened as it approached the bladder. Between the two vasa deferentia, near their distal end, was a uterus masculinus. From the caudo-medial aspect of each testicle two strands of tissue ran caudally and fused to form a ridge containing fat and smooth muscle fibres. In its last 50 mm. it acquired striated muscle fibres, fanned out to a width of 10 mm., and was finally inserted in the region of the internal inguinal ring where it was related to the cremaster muscle. This ridge was considered to be the gubernaculum. On the left side a few fibres were given off proximal

TABLE 1

Dimensions in mm. of the internal genitalia of seven bovine foetuses compared with those of a Schistosomus reflexus (S.R.).

No.	*Crown rump length*	*Testis*	*Length of seminal vesicles*	*Diameter of prostate*	*Pelvic urethra length × width × height*	*Bulbo-cavernosus muscle length × height*
C 13	285	Measurements not taken				
C 19	370	7 × 13	9	3	27 × 7 × 7	18 × 9
C 5	427	Measurements not taken				
C 4	540	18 × 19	18	4	36 × 9 × 11	24 × 16
C 9	660	10 × 23	20	4	45 × 9 × 9	30 × 12
C 55	720	12 × 20	28	—	45 × 11 × 8	29 × 11
C 53	900	16 × 27	32.5	6	45 × 13 × 11	30 × 15
C 54	950	14 × 25	37.5	6	45 × 14 × 8	30 × 17
C 56	1000	13 × 27	31	7	42 × 14 × 11	40 × 16
S.R.		15 × 30 14 × 25	35	7	27.5 × 13 × 15	33 × 18

to this termination and these ran over the medial aspect of the sartorius muscle to insert on the fascia of the adductor muscles of the thigh.

The dimensions of the accessory genitalia (prostate and bulbo-urethral glands, and seminal vesicles) are given in Table 1.

The rectum. Approximately 300 mm. of hind gut were still present in the specimen in a normal position, entering the pelvic cavity in the usual manner. The distal opening of the alimentary tract

was found in the normal position i.e. ventral to the tail root and immediately dorsal to the ischial arch. On removal of the hind gut with the genitalia an unusual feature was immediately apparent in that the pelvic muscles of the alimentary tract were grouped approximately 100 mm. from the opening. When the tract was opened this was partly explained in that 100 mm. consisted of hair-bearing skin, apparently drawn in to form an 'anal tube.' (Plate 1). A narrow zone (20 mm.) bearing stratified squamous epithelium separated the tube from the mucous membrane of the rectum.

The muscles of the pelvic viscera. The smooth muscles (retractor penis and recto-coccygeus) and the pelvic urethral muscle and its continuation as the bulbo-cavernosus muscle were normal, though the latter was divided by a groove into distinct proximal and distal parts. The other muscles were markedly abnormal and were divisible on each side into two sheets ; one originated from the pubis, the inter-pubic ligament and the region of the acetabulum and was inserted on the rectum, the other originated from the ilium, the sacrum, the tuber ischii and the fascia of the semi-membranosus muscle and was inserted on the urethra in the region of the bulbo-cavernosus muscle. The muscle associated with the rectum was inserted by its main mass just caudal to the level of the ano-cutaneous junction and at this point sent a ventral slip around the rectum to join its fellow on the other side. A bundle of fibres passed back on each side to be inserted on the 'anal tube' near its orifice. This muscle sent a slip to the smooth muscle of the retractor penis muscle and was believed to represent, in part at least, the levator ani (retractor ani) muscle. A few circularly running strands of striated muscle at the level of the ano-cutaneous line had connections with this muscle and were considered to represent the external anal sphincter. The muscle associated with the urethra was markedly asymmetrical, the left sheet being inserted on the penis at a more caudal level than the right. The most caudal part arose from the semi-membranosus fascia and the tuber ischii, covered the crus of the penis, and represented the ischio-cavernosus muscle.

The abdominal wall. The external and internal oblique and the transversus muscles were identifiable, all fading into aponeuroses. No rectus abdominus muscle could be seen. A slip of muscle, approximately 10 mm. wide and 50 mm. long, left the edge of the internal abdominal oblique muscle to fade out near the internal inguinal ring. This was presumed to be the external cremaster muscle.

Normal Foetuses

The data on the nine normal foetuses are arranged in Tables 1 to 3. The positions of the teats are given in Fig. 1.

TABLE 2

Dimensions in mm. of the external genitalia of nine bovine foetuses compared with those of a Schistosomus reflexus (S.R.).

No.	Crown-rump length	Height of scrotum	Length of penile sheath	Length of pre-penile sheath	Length of Penis		Total	Dimensions of penis	
					Bulbo-cavernosus to retractor penis	Retractor penis to apex		At bulbo-cavernosus muscle	At proximal extremity of free end
C 13	285	20	12	15	40	40	80	3 × 3	2 × 3
C 49	370	22	14	15	58	56	114	4 × 6	4 × 4
C 5	427	40	21	23	60	70	130	4 × 6	4 × 5
C 4	540	28	24	25	73	87	160	5 × 8	5 × 5
C 9	660	40	26	41	90	90	180	5 × 8	5 × 6
C 55	720	38	31	37	81	105	186	6 × 10	6 × 7
C 53	900	40	32	47	105	115	220	7 × 10	5 × 7
C 54	950	40	32	42	95	118	213	8 × 9	5 × 7
C 56	1000	40	32	55	105	120	225	8 × 12	6 × 7
S.R.		18	25	35	50	75	125	6 × 8	4 × 6

TABLE 3

Body measurements in mm. of nine bovine foetuses compared with those of a Schistosomus reflexus (S.R.).

No.	Crown-rump length	Head length	Tail length	Anus to scrotum	Scrotum to sheath orifice	Sheath orifice to umbilicus	Anus to umbilicus
C 13	285	101	71	60	50	6	116
C 49	370	132	132	113	60	5	178
C 5	427	143	125	125	70	15	210
C 4	540	170	170	120	90	15	220
C 9	660	195	210	175	125	30	330
C 55	720	210	240	175	125	35	335
C 53	900	225	310	186	135	50	371
C 54	950	230	340	210	160	40	410
C 56	1000	235	335	220	165	35	520
S.R.		255	285	50*	150	50	250*

*Measurement taken from external opening of anal tube and not from the true anus (see text).

DISCUSSION

Such parameters as could be compared with other bovine foetuses (head length, tail length) showed the Schistosomus reflexus to be at an overall stage of development consistent with full term (Table 3). The development of the pelvic genitalia suggests that hormonal factors were operating normally. From Table 1 it can be seen that the genitalia of the monster correspond more closely in development with those of the three foetuses near full term than with the others. The abnormalities observed occur in organs and processes closely associated with the ventral abdominal wall, and appear to be correlated with abnormal development of that structure.

As already described the muscles derived from the cloacal sphincter and those of the pelvic diaphragm show marked abnormalities. These muscles probably originate from the ventro-lateral divisions of myotomes S_3 – C_1 (Hamilton, Boyd and Mossman, 1945). According to Power (1948), the two groups of muscles are "inextricably associated." Popowsky (1899) and Power (1948) have described the process of differentiation of these muscles into their definitive structures in man. This process seems to have occurred only to a limited degree in the Schistosomus reflexus. Popowsky described the cloacal sphincter as "a skin muscle" and the formation of an anal tube from the perianal skin in this foetus may well be related to the abnormality of the muscles normally associated with this skin. Wainman and Shipounoff (1941) demonstrated the hormonal control of these muscles in the post-natal rat, and the differences seen between sexes suggest hormonal control of their organisation before birth. From our observations we believe that mechanical factors may also be involved.

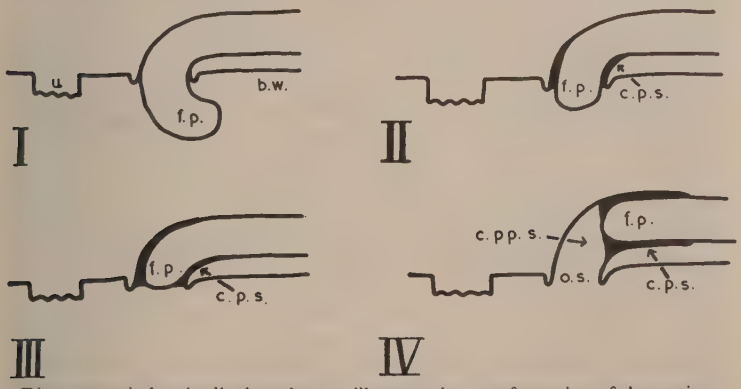
The testicles of the ox normally descend into the scrotum at the 200 to 250 mm. C.R. stage (Van Vloten, 1932) and intra-abdominal testicles in the otherwise normal full term foetus are definitely abnormal. Many of the factors which have been implicated in testicular descent are greatly changed in the Schistosomus reflexus. Differential growth of sublumbar and ventral abdominal regions was abnormal. The muscles forming the inguinal canal were reflected dorsally and the gubernaculum and processus vaginalis did not extend into the scrotum. Intra-abdominal pressure must have been influenced by failure of ventral fusion of the body walls. It is impossible to surmise which, if any, of these abnormalities was the major factor in retention of the testes in the intra-abdominal position. The statement by Williams (1943) that "most male monsters are cryptorchid" suggests that abnormal development in apparently unrelated structures may affect descent of the testicles.

Selye (1943) suggested that development of the scrotum in the rat is controlled not only by hormonal factors, but also by mechanical factors such as intra-abdominal pressure in the processus vaginalis

and presence of the testicle and epididymis in the developing scrotum. Our findings support this suggestion as, although there was no evidence of hormonal failure, the scrotum was markedly reduced in size compared with normal calf foetuses.

Failure of the body wall folds to fuse ventrally and enclose the developing viscera was accompanied by abnormal development of the region between the umbilicus and the anus. This is seen when the lengths anus to scrotum, scrotum to orifice of sheath, and orifice of sheath to umbilicus are compared with those of nine normal foetuses of varying sizes. The total length of this region in the Schistosomus reflexus corresponds with that in the 500 to 600 mm. stage of normal development and is only half of that in the 1000 mm. foetus. The major part of this reduction in total length occurred in the region between anus and scrotum; the scrotum of the Schistosomus reflexus was displaced towards the opening of the alimentary tract. The positions of the teats in normal foetuses indicate a gradual development of the abdominal wall in this region with maximum growth occurring cranially as would be expected. By comparison, the position of the teats in the Schistosomus reflexus is markedly abnormal. The skin was reflected dorsally over the body of the foetus, whose long axis was curved, the left side being convex and the right concave. Greater growth of skin occurred

Fig. 2.



Diagrammatic longitudinal sections to illustrate the transformation of the penis pendulus (I) into the penis appositus (IV) of the bovine foetus. I, 50 mm. foetus. II, 110 mm. foetus. III, 180 mm. foetus. IV, 360 mm. foetus. b.w., body wall. c.p.s., potential cavity of the penile sheath. c.pp.s., cavity of the prepenile sheath. f.p., free end of penis. o.s., orifice of the sheath. u, severed umbilicus.

over the convex side and this is shown in the asymetrical disposition of the four teats. Those on the left side are further from the midline and further apart than those on the right side. It is thought that absence of the normal even stresses imposed on the normal foetus by the developing viscera, and the presence of abnormal and uneven stresses due to dorsal reflection over a curved trunk in the Schisto-

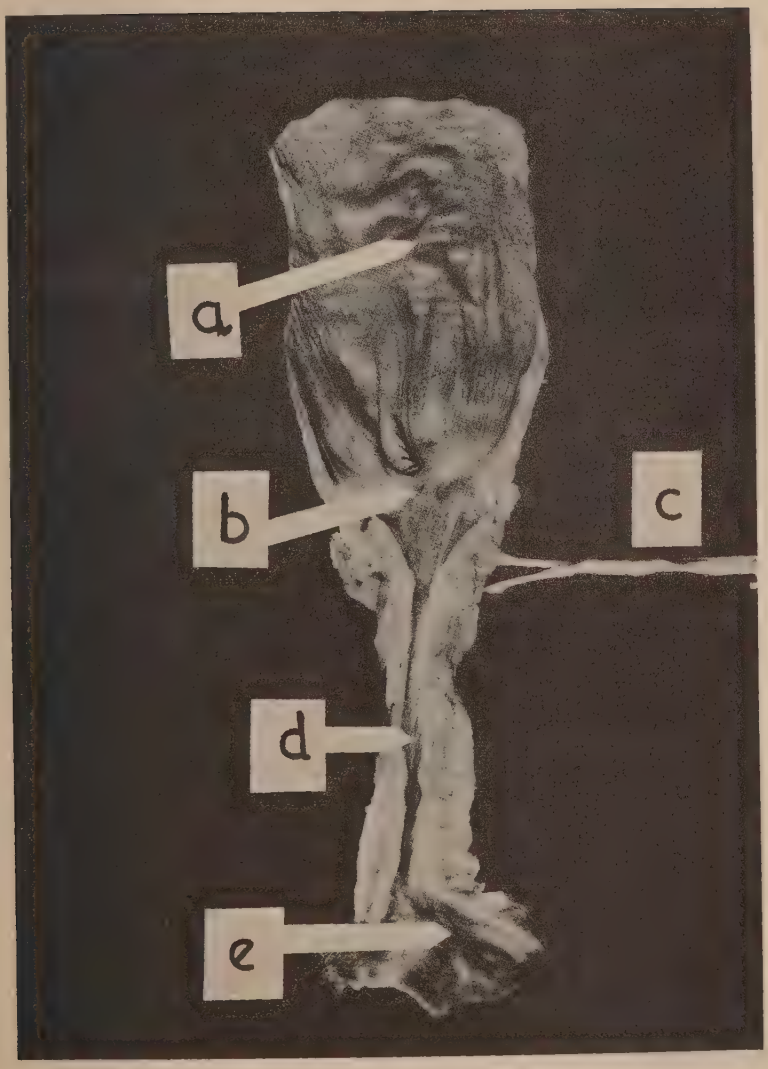
somus reflexus, were responsible for the abnormal development manifested by the ventral abdominal wall.

Early in foetal life and by the 200 mm. stage (Broman, 1947) the primitive penis pendulus is converted to the definitive penis appositus in the bovine (Fig.2). By overgrowth of skin the body of the penis becomes tied to the ventral body wall and the pars liberia penis becomes covered by the prepuce or penile sheath. The apex of the penis comes to lie at the orifice of the sheath immediately caudal to the umbilicus and covered by the penile sheath or prepuce in the 200 mm. foetus. In subsequent development a pre-penile sheath is formed so that, although by differential growth of penis and body wall the apex of the penis becomes removed from the umbilicus, the orifice of the sheath retains its position just caudal to it. This prepenile sheath is considered to be embryologically distinct from the penile sheath or prepuce (Henneberg, 1925). The differential rate of growth of penis and ventral body wall is reflected in the changing ratio of length of penile sheath to length of prepenile sheath between the 400 and 1000 mm. stages. The prepenile sheath becomes relatively longer.

The sheath and the penis of the full term Schistosomus reflexus correspond closely in size with these structures in foetuses at the 400 to 600 mm. stage of development. They are markedly reduced in overall dimensions compared with the organs of normal full term foetuses examined. Abnormal development of the ventral abdominal wall has therefore been accompanied by arrested development in length and diameter of the penis and its sheath. It is believed that the shortening of that part of the ventral abdominal wall overlying and associated with the penis and sheath resulted from its dorsal reflection. The shortening of the distance between the opening of the alimentary tract and the scrotum, together with the presence of a long hair-bearing 'anal tube,' suggests that skin lying between the ano-cutaneous junction and the scrotum has been drawn into the pelvis. This may have been an important factor in reducing the length of skin lying between the ischial arch and the orifice of the sheath. In the bovine, both the penis and its sheath have been shown to be retarded in their post-natal development by castration (Mader, 1907; Retterer and Neuville, 1916; Ashdown, 1956). Growth of the ventral abdominal wall, to which anatomically and developmentally they are closely related, also appears to influence development of the penis and sheath and has itself been shown to be influenced by mechanical stresses imposed on it during development.

The facts of teratology must be applied with caution to the study of normal development. Nevertheless, we believe that the evidence presented here shows that hormonal influences are not solely responsible for the normal development of the scrotum, penis, sheath and musculature of the genital tract of the ox but that mechanical factors are also involved.

Plate 1.



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CONCLUSIONS

The gross anatomy of the reproductive tract of a full term male Schistosomus reflexus is described and compared with the organs of nine other bovine foetuses of varying sizes. In the Schistosomus reflexus the internal genitalia were normal, while the external genitalia were under-developed. These findings are related to abnormal development of the body wall and it is suggested that mechanical factors were responsible for arrested growth of the external genitalia. The significance of this in normal development is discussed.

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Plate 1. Caudal extremity of the alimentary tract of the Schistosomus reflexus monstrosity calf. The tract has been opened dorsally to show the changing nature of the epithelium. a, mucous membrane of the rectum. b, hairless band of stratified squamous epithelium. c, retractor penis muscle. d, hair-bearing skin of the 'anal tube.' e, opening of the alimentary tract.

THE EFFECT OF FEEDING DIFFERENT LEVELS OF PROTEIN CONCENTRATES ON THE SUSCEPTIBILITY OF CHICKENS TO *SALMONELLA GALLINARUM* INFECTION

By

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INTRODUCTION

One important factor influencing the response of animals to infection is the composition of the diet, and a considerable amount of work has been carried out on the influence of it on the susceptibility of certain laboratory animals to experimental Salmonella infections. In particular Schneider and Webster (1945) and Schneider (1948) have shown that under certain conditions diet plays an important role in the resistance of mice to such infections, but few such experiments have been performed on poultry. A recent report (Hill, Garren, Kelly and Barber, 1955) has demonstrated the protective effect of high levels of vitamins against *Salmonella gallinarum* infection in young chickens. Another approach has been that of Smith (1954) who showed that the composition of the food in which *Salm. gallinarum* was administered influenced the subsequent infection rate. In the course of one such experiment the impression was gained that the level of a certain protein concentrate was affecting the mortality rate as well as the infection rate.

It therefore seemed worthwhile to investigate the subject, and the purpose of this paper is to record the effect of varying the level of certain protein concentrates in a simple cereal mixture. Most attention was directed to fish meal fed in a basal diet consisting solely of finely ground wheat. Chickens receiving such simple mixtures were then experimentally infected with *Salm. gallinarum*. The results of varying the composition of these simple mixtures at different intervals of time in relation to infection time have also been recorded.

At the outset it must be stressed that such diets have never been considered balanced in respect of the essential amino-acids, vitamins and other essential nutrients, but it is significant to record that chickens were kept for many weeks on them without any untoward effect apart from a reduction in growth rate. In view of the work of Dubos (1955), the significance of fasting chickens before infecting them with *Salm. gallinarum* has also been investigated.

In experimental *Salm. gallinarum* infection, chickens may die during the acute bacteraemic phase or during the chronic phase when they are emaciated and anaemic. In the latter case, death

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probably results from the presence of large proliferative lesions principally in the myocardium and intestinal wall (Smith, 1955). In the breed of chickens used in the present investigation, deaths of the acute type occur within 14 days of infection and of the chronic type usually after that time, the mortality, however, being very low after 21 days. In view of the essential dissimilarity of the two forms of the disease, the results of the experiment described below are classified according to the total mortality and to the mortality from the acute form of the disease.

MATERIALS AND METHODS

Chickens. Sex-linked Brown Leghorn × Barred Rock cockerels from a Salmonella—free flock were used.

Diets. The basis of all experimental diets was finely ground wheat. The white fish meal was an ordinary commercial preparation of the best quality with a crude protein content of 63 per cent. and an ash content of 20 per cent. The decorticated ground-nut meal was a normal commercially-produced product containing approximately 51 per cent crude protein, 5·2 per cent ash, and 5·7 per cent fibre.

The dried skim milk was also an ordinary commercial preparation with a protein content of 35 per cent and an ash content of 7·8 per cent.

Two practical rations were used for rearing the chickens and for some of the experimental studies. They had the following composition :—

	Mash A. Per cent.	*Mash B.* Per cent.
White fish meal	5	10
Dried skim milk	5	0
Decorticated ground-nut meal	5	5
Maize	11¾	10
Wheat	20	15
Barley	20	17
Sussex ground oats	10	15
Wheat middlings	15	19
Grass meal	5	5
Unextracted yeast	2	2½
Minerals	1¼	1½
Synth. Vitamin A	2000 I.U./lb.	2000 I.U./lb.
Synth. Vitamin D_3	446 I.U./lb.	446 I.U./lb.
Riboflavin	1 mg./lb.	1 mg./lb.

Food and water in all experiments were provided *ad libitum.*

Experimental procedure. For the first five weeks of life all chickens were kept in wire-floored brooders and fed entirely on chick mash A. They were then given the experimental diets for 21 days, when they were infected. In some of the experiments changes of diet were made at, or three days before, infection. At 5 p.m. on the day before infection all food was withdrawn from the brooders. Early the following morning the chickens were moved to raised arks with slatted floors and infected by mouth at approximately 10 a.m. The chickens were allowed access to food about 15 minutes later. They were then kept under observation for a further 21 days and the daily mortality recorded during this period.

Autopsies were performed on all chickens that died ten or more days after infection to determine whether they had died from the acute or chronic form of the disease.

Method of producing infection. This has been described elsewhere (Smith, 1955). The method was evolved because previous work (Smith, 1954) had indicated that the diet of chickens, through its influence on the bactericidal action of the gastric juice in the gizzard, could affect the numbers of *Salm. gallinarum* passing through that organ. Bearing in mind that quite different diets were being studied in the present work, it was thought that a standard infective dose using the oral route could best be obtained by depriving chickens of food overnight to ensure a relatively empty gizzard and by giving alkali with the *Salm. gallinarum* to protect the bacteria from the lethal effect of the residual acid.

RESULTS

The Effect of Different Simple Practical Diets on the Course of Infection.

The results of infecting with *Salm. gallinarum* groups of 75 chickens fed diets consisting of finely ground whole wheat with or without various protein concentrates for 3 weeks prior to infection and during the course of the disease are shown in Table 1. The survival time was longest and the mortality rate lowest in the group fed ground wheat plus 2½ per cent fish meal. The severity of the disease increased progressively as the amount of fish meal increased above this level and the amount of ground wheat correspondingly decreased ; the total mortality in the groups fed 2½, 10 and 40 per cent fish meal was 52·0, 70·7 and 97·3 per cent respectively and all the chickens in the 40 per cent fish meal group that died except two did so in the acute phase of the disease. On the other hand, the complete absence of fish meal, i.e. a diet consisting solely of ground wheat, did not result in a milder form of the disease. In fact, the reverse occurred with 82·7 per cent of the chickens dying, a considerable proportion of the mortality occurring early in the disease. The course of the disease in the chickens fed ground wheat plus 50 per cent decorticated groundnut meal was similar to that which occurred in the chickens fed ground wheat plus 40 per cent fish meal. Both of these diets had approximately the same crude protein content, namely, 32 per cent. A slightly lower mortality rate of 60 per cent was observed in the group of chickens fed ground wheat containing 10 per cent fish meal plus 10 per cent dried skim milk than in the group that received the diet of wheat supplemented with 10 per cent fish meal only.

Similar experiments to those described above, but using smaller groups of chickens, were carried out on other occasions with substantially similar results.

The Effect of Changing the Diet immediately after Infection

The results of changing the diet of groups of 45 chickens from one containing a high level of fish meal (40 per cent) to one containing a low level (10 per cent) or none at all (ground wheat only)

TABLE 1

THE EFFECT ON INFECTION OF FEEDING BASAL DIET

Groups of 75 Chickens receiving a Basal Diet of Fine Ground Wheat Containing Different Proportions of Two Different Protein Concentrate Supplements

Protein supplement	*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
	5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	%	*No.*	%
None	4	14	24	33	45	49	49	52	54	58	58	60	62	62	82·7	55	73·3
Fish meal, 2½%	0	0	1	5	11	19	23	25	30	32	34	35	39	39	52·0	30	40·0
Fish meal, 10%	0	0	2	10	24	30	35	39	40	46	51	51	53	53	70·7	43	57·3
Fish meal, 40%	5	15	32	54	62	67	68	71	71	71	71	72	73	73	97·3	71	94·7
Decort. groundnut meal, 50%	13	34	48	54	62	65	66	67	71	72	73	73	73	73	97·3	72	96·0
Fish meal, 10%, and dried skimmed milk, 10%	0	1	3	7	19	25	31	36	37	39	39	40	45	45	60·0	39	52·0

TABLE 2

THE EFFECT ON INFECTION OF CHANGING THE DIET

Groups of 45 Chickens Previously Fed Finely Ground Wheat + 40 per cent Fish Meal to Other Diets at Infection Time

New diet	*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
	5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	%	*No.*	%
Ground Wheat	0	0	3	8	12	19	26	29	33	34	36	37	39	39	86·7	31	69·0
Ground wheat + 10% fish meal	0	0	6	15	23	30	32	34	35	36	36	39	42	42	93·3	35	77·7
No change (i.e. ground wheat + 40 % fish meal)	0	2	5	18	26	31	36	38	40	41	42	42	42	42	93·3	39	86·7

immediately after they were infected with *Salm. gallinarum* are shown in Table 2. As a control, a group of chickens was given the high-level fish meal diet throughout. No apparent difference was observed between the course of the disease in the group changed from the high level fish meal diet to the low level diet compared with the group maintained continually on the high level fish meal diet. On the other hand, the change to a diet solely of ground wheat prolonged the survival time of the chickens in this group. The total mortality rate, however, was not subsequently altered.

When the above experiment was repeated using groups of 36 chickens whose diet prior to infection was ground wheat plus 10 per cent fish meal instead of 40 per cent fish meal, there was little or no difference between the total mortality rate in all three groups (Table 3). Deaths occurred more frequently, however, in the early stages of the disease in those chickens whose diet after infection contained the high level of fish meal than in those of the other two groups.

The total mortality rates in each of these above two experiments were similar to those observed when the diet provided before infection was given continuously throughout the experiment (Table 1).

The Effect of Changing the Diet Three Days before Infection

The effect of making alterations in the diet of groups of 30 chickens three days before they were infected with *Salm. gallinarum* is illustrated in Table 4. The results were much more striking than when changes were made at the time of infection as in the previous experiments. The disease was much less severe in the group of chickens whose diet was changed from ground wheat plus 40 per cent fish meal to ground wheat only than it was in those fed either ground wheat or ground wheat plus 40 per cent fish meal throughout the experiment. This was particularly noticeable when the acute phase of the disease was considered, only 30 per cent of the former group dying in this phase compared with 70 per cent and 93 per cent respectively in the other two groups. Altering the diet in the reverse direction, i.e. from ground wheat to ground wheat plus 40 per cent fish meal did not have a similar effect ; a very severe disease was, in fact, produced with an 86·7 per cent mortality in the acute phase. A marked prolongation of survival time and a reduction in death rate, particularly from the acute form of the disease, was also noted in the group of chickens whose diet was changed from ground wheat plus 10 per cent fish meal to ground wheat only compared with the group maintained on the diet containing 10 per cent fish meal throughout. It is noteworthy that in the groups of chickens whose diet was changed to ground wheat only the greater proportion of deaths occurred in the chronic phase of the disease.

In a similar experiment in which the fish meal addition of either

TABLE 3

THE EFFECT ON INFECTION OF CHANGING THE DIET

Groups of 36 Chickens Previously Fed Finely Ground Wheat + 10 per cent Fish Meal to Other Diets at Infection Time

New diet	*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
	5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	*%*	*No.*	*%*
Ground wheat	0	1	2	7	12	17	20	22	22	22	23	23	23	23	63·8	22	61·1
Ground wheat + 40% fish meal	1	5	10	17	20	22	22	23	23	23	24	24	25	25	69·1	23	63·8
No change (i.e. ground wheat + 10% fish meal	0	3	3	13	14	20	21	21	21	21	22	22	23	23	63·8	21	58·3

TABLE 4

THE EFFECT ON INFECTION OF ALTERING THE AMOUNT OF FISH MEAL

Fed to Groups of 30 Chickens Three Days Before Infection

Per cent of fish meal in		*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
Previous diet	*New diet*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	*%*	*No.*	*%*
40	40	0	4	10	16	25	28	28	28	28	28	28	29	30	30	100	28	93·3
40	0	0	0	0	0	1	3	5	6	8	10	14	17	19	19	63·3	9	30·0
0	0	2	3	9	12	15	15	17	18	21	21	21	22	24	24	80·0	21	70·0
0	40	4	12	19	22	24	24	24	24	25	27	28	28	28	28	93·3	26	86·7
10	10	0	0	2	5	8	10	14	16	17	18	18	18	20	20	66·7	17	56·7
10	0	0	0	0	0	2	4	5	7	8	9	10	12	18	18	60·0	8	26·7

* The only other constituent of the diet was finely ground wheat.

TABLE 5

THE EFFECT OF STARVATION

Groups of 36 Chickens Starved Before Infecting them by Mouth

Starvation period (hours)	*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
	5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	*%*	*No.*	*%*
48	3	5	13	23	28	30	30	30	31	31	31	31	31	31	86·1	31	86·1
24	0	2	4	10	21	22	26	27	28	28	28	29	30	30	83·3	28	77·7
16	0	2	7	13	20	23	27	28	28	28	29	30	30	30	83·3	28	77·3

TABLE 6

THE EFFECT OF STARVATION

Group of 30 Chickens Starved for 48 Hours Before Infection Subcutaneously

Infection	*Total no. of chickens dead at the following days after infection*													*Total mortality*		*Mortality from acute form*	
	5	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17+*	*No.*	*%*	*No.*	*%*
Starved	2	5	9	13	16	18	19	20	20	22	22	24	24	24	80	22	73·3
Not starved	2	6	11	15	19	19	21	23	25	25	25	25	26	26	86·6	25	83·3

The dose of *Salm. gallinarum* employed was approx. 10^7 viable bacteria.

10 or 40 per cent was reduced to 2½ per cent three days before infection, an effect was noted similar to that observed in the previous experiment when the new diet consisted solely of ground wheat.

The Effect of Starvation on Infection

The results of starving groups of 36 chickens for different periods of time before infecting them by mouth is shown in Table 5. Very little difference was noted between the three groups as regards mortality, but the survival times were shorter in the group fasted for 48 hours than in the groups fasted for 16 and 24 hours. All the chickens in these groups and those used in the next experiment were maintained on mash B. When the subcutaneous, instead of the oral, route of infection was employed, no difference was noted in the course of the disease in two groups of 30 chickens, one of which had been fasted for 48 hours before infection and one which had not. (Table 6).

DISCUSSION

Any real appraisal of the role played by nutrition in resistance to infection must involve a consideration of many different factors. The ideal experimental approach from the point of view of the nutritional status of the host is one in which diets of highly purified constituents are used and individual deficiency states produced. However, in the field a simple deficiency of one nutrient rarely occurs and here one is dealing with adequate and inadequate diets composed of many combinations of individual nutrients in varying amounts and, under such conditions, it becomes almost impossible to assess their role in nutrition.

There is as yet little published work on specific nutritional factors which can influence *Salmonella* infection in poultry. The main purpose of our work has been to make a start by using simple practical diets and varying only one major component. No attempt has been made to provide optimal requirements of the vitamins, minerals and essential amino-acids.

Our results indicate that, of the diets studied, finely ground wheat plus 2½ per cent fish meal provided the highest degree of resistance to infection. High levels of this protein concentrate or its total absence had an adverse effect on the course of the disease. As far as high levels of fish meal were concerned, it is probable that its protein moiety was responsible for the adverse effect since similar results were obtained with a diet containing an alternative source of protein concentrate, namely, decorticated ground-nut meal. Previous work (Smith, 1954) demonstrated that chickens could be more easily infected *per os* with *Salm. gallinarum* when they were fed diets containing high levels of fish meal since the pH of the gizzard of such chickens was far too high to have any significant effect in destroying *Salm. gallinarum* contained therein. The present studies

have indicated that the disease, once contracted, is likely to be severe and will probably terminate fatally if the feeding of high levels of fish meal is continued. Whether these results may have general application to other diseases of poultry is not known. It is noteworthy, however, that Mann (1945) in field observations noted higher mortality rates from *Salm. pullorum* infection in chicks fed high levels of protein than in chicks fed lower levels.

The results of changing the diet either at or three days prior to infection indicate that in flocks in which an outbreak of fowl typhoid has occurred, the diet might with advantage be altered to one containing comparatively low amounts of protein concentrate, particularly fish meal, while other methods of control are being instituted. This may well have a beneficial effect as field outbreaks are not usually explosive in character but spread slowly compared with many other diseases.

The results of changing the diet of infected chickens were much more noticeable when they were made three days before, than at infection time. The explanation of this may be a simple one concerned with appetite, infected birds having a smaller appetite than healthy ones (Smith, 1955). The effects of these changes throw some light on the manner in which diet may influence the course of the disease since it is highly improbable that they were made soon enough to alter the general nutritional status of the host. The continuous feeding of either ground wheat or ground wheat plus 40 per cent fish meal commencing three weeks before infection had an adverse effect on the course of the disease. A similar effect was noted when the diet of ground wheat was changed to one of ground wheat plus 40 per cent fish meal. On the other hand a change in the reverse direction had a very beneficial influence. It is probable then that the mode of action of these two diets was quite different. The adverse effect of the high level protein diets was most likely due to the great stress thrown, for example, on the liver during their metabolism, this organ also being concerned with defence against infection. Such an effect would be relatively immediate and, consequently, would account for the high mortality experienced when the changes to high protein diets were made shortly before infection. The two different and opposite effects obtained with the diet of ground wheat only, depending on whether it was fed throughout or whether it was substituted for a high protein diet shortly before infecting, cannot be explained on this basis. It is conceivable, though, that ground wheat may be deficient in some factor associated with resistance to fatal infection that is present in fishmeal. This would account for the adverse effect of this diet when feeding is commenced 21 days before infection and also for the beneficial effect when commenced three days before infection if it is assumed in the latter case the tissues of the chickens submitted to this dietary regime contain sufficient of this hypothetical factor obtained from their previous dietary regime which contained fish meal. These

explanations, too, accord with the fact that ground wheat containing 2½ per cent fish meal was the most satisfactory diet as regards resistance to *Salm. gallinarum* infection. It is noteworthy that counts of *Salm. gallinarum* performed twice daily on the blood and faeces of infected chickens that had been fed a diet of either ground wheat alone or with added fish meal (at either 2½ or 40 per cent) revealed a similar bacteriological picture irrespective of the diet. The bacteraemic phase commenced at approximately the third day after infection, and high counts were observed shortly before death which, of course, occurred later in chickens fed the diet containing 2½ per cent fish meal than in those fed the other two diets.

The results of infecting starved and normally fed chickens parenterally do not indicate that the observations of Dubos (1955) have general application. This worker found that the course of bacterial diseases in mice produced by the parenteral route was more severe in animals that had been starved before infection than in animals which had been normally fed right up to the time of infection. As regards oral infection, however, the course of *Salm. gallinarum* infection appeared to be somewhat more severe in chickens fasted for 48 hours than in chickens fasted for shorter periods. It is known that a period of 48 hours is often necessary before the alimentary tract, and particularly the gizzard, becomes completely empty and it is conceivable that this fact, together with the possibility that changes may occur in the alimentary tract after 48 hours which facilitate the entrance of *Salm. gallinarum*, may account for this observation.

CONCLUSIONS

The results of feeding chickens diets of ground wheat containing different amounts of fish meal commencing three weeks before infection indicated that the diet which produced the greatest resistance to *Salm. gallinarum* infection was one of ground wheat plus 2½ per cent fish meal. The absence of fish meal, or its addition at higher levels (10 and 40 per cent), had an adverse effect on the course of the disease. A high level of decorticated groundnut meal (50 per cent) produced a similar effect.

Changing the diet from one of ground wheat containing high levels of fish meal to one composed of ground wheat only at, or preferably three days before, infection had a beneficial influence on the course of the disease. This was not true of dietary changes in the reverse direction.

Fasting chickens for 48 hours before infection had no observable effect when infection was produced parenterally. When produced orally, however, the disease was somewhat more severe in chickens fasted for 48 hours than in chickens fasted for shorter periods of time.

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THE INTRAOCULAR LESIONS OF CANINE DISTEMPER

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INTRODUCTION

The observation that blindness might occur as a sequel to distemper or as an accompaniment of nervous distemper dates back at least to Coats (1913), who described the histologic changes in the retina of a dog which became blind following an attack of distemper. Coats noted that during life both pupils had remained widely dilated. Histologically, he found degeneration of the ganglion cells and atrophy of the inner reticular and outer nuclear layers. In certain local areas there were more severe lesions, consisting of layer disorganization with complete loss of rods and cones, proliferation of pigment epithelium and pigmentary invasion of the retina.

Subsequent to this, there were occasional clinical observations of blindness as a part of the distemper syndrome, none of which appeared to stimulate any investigation of a possible anatomic basis for the condition. (Carré, 1921; Kirk, 1922; Goldberg and Volgenau, 1925). During this period, and the ensuing three decades, the histopathology of the brain in distemper infection was actively studied, and several workers reported lesions in the optic chiasm and optic tracts (Winqvist, 1950). The paper by Potel (1953) on the histopathology of nervous distemper reviewed 121 publications, none of which dealt with intraocular changes. In describing the symptoms of the dogs in their series Goldberg and Volgenau stated: "Derangement of sight occurred in the form of so-called amaurosis, that is, no visible macroscopical lesion was observed. This type of blindness may end in sudden recovery, partial blindness, or total permanent blindness in one or both eyes. The pupil was invariably widely dilated and did not readily respond to myotics." They reported detailed histologic observations on the neuropathology in their cases, but did not supplement their observations of the eyes with histologic studies. Robin and Brion (1939) stated that they had observed retinitis in canine distemper, but did not say whether ophthalmoscopically or histologically and gave no details.

Parry (1954) was the next to report on the histopathology of the eye in canine distemper subsequent to Coats' original description. In a study of 24 dogs he observed primary chorio-retinal lesions which he divided into four types:—"(1) A peracute generalized retinopathy occurring during the initial stages of acute encephalopathy; (2) a chronic generalized retinopathy in animals showing delayed neurological sequelae, so-called post-infective (distemper) encephalopathy (Parry, 1957); (3) a dystrophy of the cells of the pigment epithelium with or without evidence of other damage to the retina; (4) sporadic foci of advanced degeneration of the retina, focal retinal atrophy, and sclerosis."

Although various experimenters have injected viruses into animals' eyes, there are few descriptions of the resulting histopathology. In most instances, the only lesions described were gross ones, and even these were

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mentioned only when they involved the cornea. The encyclopaedic treatises of virology, such as Doerr's, and of ophthalmology such as Duke-Elder's have little information on the effects of viruses on the intraocular tissues, except for those that incite macroscopic lesions.

One of the earliest reports was that of Goodpasture and Teague (1923), who studied the eyes of rabbits following intraocular infection with the virus of herpes simplex. They found retinitis and optic neuritis which extended to the chiasma. The meninges surrounding the optic nerve were infiltrated with a mononuclear cell exudate. Typical herpes inclusion bodies were present in cells (unspecified) of the retina and in glia cells in the optic nerve.

Nicolau, Dimancesco-Nicolau and Galloway (1929) injected Borna disease virus intraocularly into rabbits. They found inclusion bodies in the retinal ganglion cells, which finding was later confirmed by Stofer (1934). Gmelin (1931) described similar inclusion bodies in naturally infected horses, in which he found also infiltrative foci of choroiditis and retinitis. Stofer (1934) studied experimental Borna disease in rabbits, sheep and horses. He demonstrated inclusion bodies in the retinal ganglion cells of all three species, but retinitis was a prominent feature only in the rabbits and sheep. He concluded that the intraocular lesions of natural Borna disease in horses could be distinguished histologically from those of periodic ophthalmia by the absence of uveitis in Borna disease. More recently Walther (1952) reported non-suppurative retinitis and optic neuritis in a natural case of Borna disease. The findings of other investigators on the effects of viruses on the intraocular tissues are summarized briefly in Table 1.

Parry (1954) found lesions in both natural and experimental distemper cases. Although his paper appeared while our study was in progress, the presence of lesions in our cases not described by him seemed to warrant its continuation. In addition, we wished to compare the lesions of canine distemper with those caused by other viruses in the eyes of animals.

MATERIALS AND METHODS

The subjects of the present study were 25 dogs affected with distemper, 20 with nervous symptoms and five with symptoms of systemic distemper unaccompanied by nervous signs. They were collected over an 18 month period extending from 1953 to 1955. The criteria for selection were that there be clinical evidence of distemper and that the animals be made available for euthanasia immediately prior to autopsy. All of them were destroyed by the intravenous injection of pentobarbital sodium and the eyes were removed and fixed in Zenker-acetic solution in less than 5 minutes after death. Following dehydration, the lateral callotes were removed by cutting in a plane parallel to the vertical meridian and perpendicular to the posterior ciliary artery, so that each section included both tapetal and non-tapetal fundus. The callotes embedded in paraffin and the eyes in celloidin were stained with hematoxylin-eosin, cresyl violet, Weil's method for myelin sheaths and Masson's trichrome stain. In addition, the brains were examined histologically with particular reference to the optic nerves, chiasma and tracts.

Investigators	*Virus and animal species*	*Results*
Komiyama (1932)	variola, rabbits	Virus remained viable through eight serial passages; no mention of lesions.
Remlinger and Bailly (1933) ..	pseudorabies, rabbits	Keratitis only, no histologic involvement of retina.
Sabin and Olitzky (1937)	vesicular stomatitis and eastern equine encephalomyelitis, mice	Retinal lesions produced by each virus, no details reported.
Evans and Rickard (1945) ..	human influenza, rabbits	Keratitis, no lesions in other ocular tissues.
Evans and Bolin (1946)	western equine encephalomyelitis, rabbits	Keratitis and iritis, no extension of inflammation to choroid or retina. Lesions attributed to toxic action, since virus did not grow to any extent in the eye.
Evans, Dowell and Green (1950) ..	fox encephalitis, foxes, dogs and cats	Oedema of cornea, inclusions in corneal epithelial cells in all three species.
Evans and Moore (1950)	herpes, rabbits	Inclusion bodies in all of the neural elements of the retina.
	feline agranulocytosis, cats	Inclusion bodies in fibroblasts of iris and ciliary body, pigment epithelium of iris, ciliary body and retina and in bipolar cells of retina.
	ornithosis, rabbits	Oedema of cornea, numerous elementary bodies in smears of anterior chamber fluid and in corneal endothelial cells.
Focosi and Scalfi (1953)	Newcastle disease, rabbits	Keratitis and iritis, no extension of inflammation to choroid or retina. Same interpretation as Evans and Bolin, above.
Zuikova (1951)*	contagious agalactia, sheep and goats	Iridocyclitis and uveitis in most cases. Focal adhesive chorioretinitis with retinal atrophy in the later stages.
Overman and Dortch (1951) ..	Semliki forest virus, rabbits	Retinitis and mitosis in visual cells of the outer nuclear layer.
Stenius (1952)*	malignant catarrh of cattle	Keratitis and iritis.
Bruna (1953)	mumps, rabbits, rats and guinea pigs	Non-suppurative optic neuritis in rabbits; no lesions in rats and guinea pigs.

*Zuikova and Stenius studied the spontaneous disease in its natural host. The other reports deal with experimental infection established by ntraocular inoculation.

In one case the diagnosis was established by isolation of the virus from the brain. In all of the others the diagnosis was made by finding inclusion bodies in the cerebral astrocytes, in urinary bladder, renal pelvic or bronchial epithelium or in all of these sites. In three cases retinal lesions were present along with inclusion bodies in the viscera but without clinical or anatomical involvement of the central nervous system.

Eye sections from 12 normal dogs and from 10 aged dogs were available as controls. Menner's work (1939) was used as a histologic guidepost. He discovered that there are normally considerable variations in both the thickness and the cytology of the retinal layers amongst various breeds of dogs. His work indicates an obvious need for caution in describing atrophic changes in the retina and our studies were carried out with this in mind.

RESULTS

With the exception of two cases, all of the eyes showed varying degrees of degenerative change in the retinal ganglion cells. This consisted of dispersion of the Nissl substance and peripheral migration of the nuclei. In many ganglion cells this migration had carried the nucleus partly out of the normal contour of the cell (Fig. 1). In some of the eyes with ganglion cell degeneration the optic nerve presented demyelination, whereas in others the optic nerve appeared unaffected. Optic neuritis, consisting of myelin destruction and proliferation of astrocytes was present in both cases which had inflammatory lesions in the optic chiasma and tracts and in cases which did not (Figs. 9, 10). Intranuclear, eosinophilic inclusion bodies in the astrocytes were seen in the optic nerve near the papilla in three cases. They were present in glial cells in the ganglion cell layer of the retina in six cases and in a cell of the inner nuclear layer in one case (Figs. 4-7).

In three cases, where the optic neuritis was histologically of a chronic nature, the chronicity could be correlated with an extended clinical course, in which the duration of the nervous symptoms was three to four weeks. In these cases there was a pronounced increase in astrocytic glia in the optic nerve and papilla (Fig. 12) and in one of them there was also a proliferation of fibroblasts in the optic papilla, the identity of which was confirmed in Masson-stained sections. Gliosis was present in the optic fibre and ganglion cell layers of these eyes and also in four cases in which there was no optic nerve gliosis.

Atrophy of the retina without loss of layer organization was seen in three cases, involving only the layer of rods and cones; the lesions consisted of degeneration and shortening of the outer limbs and pyknosis of the nuclei (Fig. 3). Atrophy of the retina with loss of layer organization was seen in ten of the cases. In some of them the atrophy was restricted to relatively small zones, sharply demarcated,

the remainder of the retina having a normal architecture (Fig. 11). In others, for which there was usually a history of protracted symptoms, the whole of the retina was affected and the atrophy included both the central and the peripheral zones, the tapetal and the non-tapetal fundus. The atrophy with loss of layer organization included in addition to the rods and cones both nuclear layers and both reticular layers. In the final stages of this process, disappearance of the ganglion cells was the striking feature, so that the retina consisted only of a layer, two to six cells deep, which appeared to be a remnant of the inner nuclear cells (centre of Fig. 11). The choroid underlying areas of retinal atrophy was also atrophic.

Swelling of the pigment epithelial cells of the retina was observed in eleven of the cases and was more marked over the non-tapetal than over the tapetal fundus. In more severe instances of pigment epithelial involvement there was proliferation of these cells in localized zones, causing detachment of the rods and cones from the external limiting membrane.

Clumps of melanin pigment lying just under the internal limiting membrane were seen in each case in which there was extensive retinal atrophy with loss of layer organization (Fig. 11). This pigment was also present in some of the cases of atrophy in which layer organization was preserved. We have seen such pigmentary invasion of the retina at the ora serrata of most old dogs not affected with retinal disease and in these it is obviously a senile change. In the distemper dogs, however, the pigmentation was often present in the central as well as the peripheral retina and was seen in animals six months of age and younger. It was also present in the pars ciliaris retinae of these young dogs. There were cases in which the above-mentioned proliferating retinal pigment epithelium could be seen invading the inner layers of the retina and in these the source of the melanin was obvious. In other cases, cells could not be clearly distinguished in the clumps of melanin, even in bleached sections, but their smoothness of outline, both round and ovoid, suggested that they might have been deposited by pigment-bearing cells.

Acute retinitis, characterized by congestion and perivascular cuffing of blood vessels in the optic nerve and ganglion cell layers, was found in six of the cases. The cuffs were composed of pale-staining mononuclear cells with round nuclei and scanty cytoplasm. In these retinas there was also oedema of the optic nerve layer with separation of the inner limiting membrane (Fig. 2). In two cases the oedema involved all of the layers of the retina. Three of the cases exhibited prominent papilloedema. In four cases, an abnormally high percentage of leukocytes was seen in the scleral veins, in the central retinal vein and in one case in the veins of the iris. One of the eyes in which these acute inflammatory lesions were most extensive came from a dog in which neither lesions nor inclusion bodies were found in the brain and the diagnosis was established by

the demonstration of inclusion bodies in the bronchial epithelium. No significant changes were seen in the cornea, iris, ciliary body or lens.

DISCUSSION

The finding of perivascular cuffing and inclusion bodies in the retina is perhaps to be expected in a viral infection; however, these lesions have not previously been reported from natural viral infection in the dog. Amongst natural infections in other animals, somewhat similar lesions have been reported only in Borna disease of horses, in which the inclusion bodies are in the retinal ganglion cells; and in contagious agalactia of sheep and goats in which no inclusions have been found. In experimental pathology, the similar findings are those of Friedenwald and McKee (1938), who found retinitis with perivascular cuffing in dogs inoculated with an unidentified filter-passing agent from a human eye; and of Goodpasture and Teague (1923), who worked with herpes simplex in rabbits.

In our distemper cases, the occurrence of retinal lesions in the presence of systemic viral infection poses two questions. (1) Is the lesion due to the presence of virus in the retina, either by primary localization concurrently with localization in other organs or by secondary extension from the brain along the optic nerve? (2) Are the retinal lesions of a retrograde degenerative nature secondary to involvement of the optic nerves? The observations recorded here permit an affirmative answer to both questions. Furthermore, there is evidence in some cases for the co-existence of retrograde degenerative lesions and lesions due directly to the presence of virus in the retina.

Evidence for the presence of virus in the retina was the finding of inclusion bodies in the retinal glial nuclei, entirely analagous to those typically present in the cerebral glia. The primary localization of virus in the retina in some of the cases is shown by the presence of an acute retinitis in the absence of detectable brain or optic nerve involvement. Focal lesions involving all layers of the retina indicate the presence of a local destructive influence (virus); retrograde degeneration does not pass across synapses, i.e., in the direction from the ganglion cells to the inner nuclear layer. We are aware of only one exception to this generalization, the cells of the lateral geniculate body, in which "transneuronal atrophy, following section of optic nerves, has been demonstrated without doubt." (Cook, Walker and Barr, 1951). Furthermore, Overman and Dortch (1951) were able to produce focal retinitis by the intraocular injection of Semliki forest virus. Our data do not indicate whether the alternative possibility of extension of infection from the brain may occur. From the basis of the known mechanics of virus infection it can reasonably be inferred that it does indeed occur.

The presence of degenerative lesions in the retinal ganglion cells

in association with optic neuritis and unassociated with retinitis is strongly suggestive that retrograde degeneration does occur in some of these distemper cases. On the other hand, it has been demonstrated that other viruses, viz., those of Borna disease (Stofer, 1934), equine encephalomyelitis and vesicular stomatitis (Sabin and Olitzky, 1937), when injected intraocularly can produce ganglion cell degeneration without retinitis.

The proliferation of the pigment epithelium, described by Coats (1913), Parry (1954) and ourselves is not unique to distemper, but is apt to occur in various inflammatory or degenerative diseases of the retina. Noel (1952) and Karli (1954) described similar lesions in cats and in rabbits that had been poisoned by the administration of iodoacetic acid, and Jaensch and Lerche (1933) in chickens affected with ocular leukosis. In human ophthalmology the situation is probably best summed up by Reese (1951) who stated that "The pigment epithelium of the retina proliferates on the slightest provocation."

The difficulties of distinguishing clinically retinal atrophy secondary to distemper retinitis from primary (hereditary) forms have been emphasized by Magrane (1955), who stressed that every effort must be made to exclude distemper by careful history taking. That this may not always be easy or even possible is illustrated by a case seen in a one year old Dachshund early in 1956. The animal was owned by a veterinarian who had raised her from a pup and immunized her against distemper and infectious hepatitis. She exhibited anorexia and mild fever for 48 hours, following which her appetite returned. Two weeks later impaired vision, a green reflex and constant mydriasis of both eyes suddenly attracted the owner's attention and our ophthalmoscopic examination at this time revealed a mild bilateral papilloedema. No neurologic symptoms other than mydriasis were noted at any time and, except for the transient anorexia, there were no signs of systemic involvement of any kind. Sudden onset of ocular symtoms, under conditions of close professional observation, is a clinical feature not associated with retinal atrophy of the hereditary type. If we assume, although we cannot prove, that the dog had a mild attack of distemper infection, it is readily apparent that many owners would not have noticed the eye symptoms as soon as the veterinarian did, and by the time they did, they would have forgotten the anorexia, or not associated it with the blindness and not mentioned it to the person taking the history.

The differentiation is not so difficult by histological examination, for, as has been shown, the virus infection is characterized by necrosis of the ganglion cells, whereas in the various hereditary atrophies it is not unusual to find intact ganglion cells in retinas which are atrophic and have lost all semblance of their normal layer organization (Parry, 1953). It is well known that in attempting to establish a post mortem diagnosis of distemper, lesions are not found in any one organ with predictable regularity. In the present study, retinal

lesions of one kind or another were found in all cases of distemper examined. If this observation remains consistent in an extended series of cases, it would provide an exception to the above rule. Not all of the dogs in this series showed signs of clinically detectable blindness; however, the presence of the lesions described provides an anatomic basis, additional to the cerebral and optic tract lesions, for the frequent clinical observation that blindness occurs in this disease. The assumption made by Kirk (1922), that the blindness is "probably a sequel to retinitis . . ." has been confirmed.

CONCLUSIONS

In canine distemper infection of dogs, intraocular lesions may be present concurrently with or in the absence of brain lesions. If the brain is involved, there may be retrograde degeneration of the retinal ganglion cells via the optic nerve. The lesions found were: degeneration of retinal ganglion cells, oedema of the retina, perivascular cuffing of retinal blood vessels, intranuclear eosinophilic inclusion bodies in retinal and optic nerve glial cells, swelling and proliferation of the retinal pigment epithelium, local or widespread atrophy of the layer of rods and cones, atrophy of all of the retinal elements with loss of layer organization, focal gliosis, and invasion of the atrophic retina by black pigment. Some of these lesions resembled those reported in the eyes of other animals with natural or experimental viral infections.

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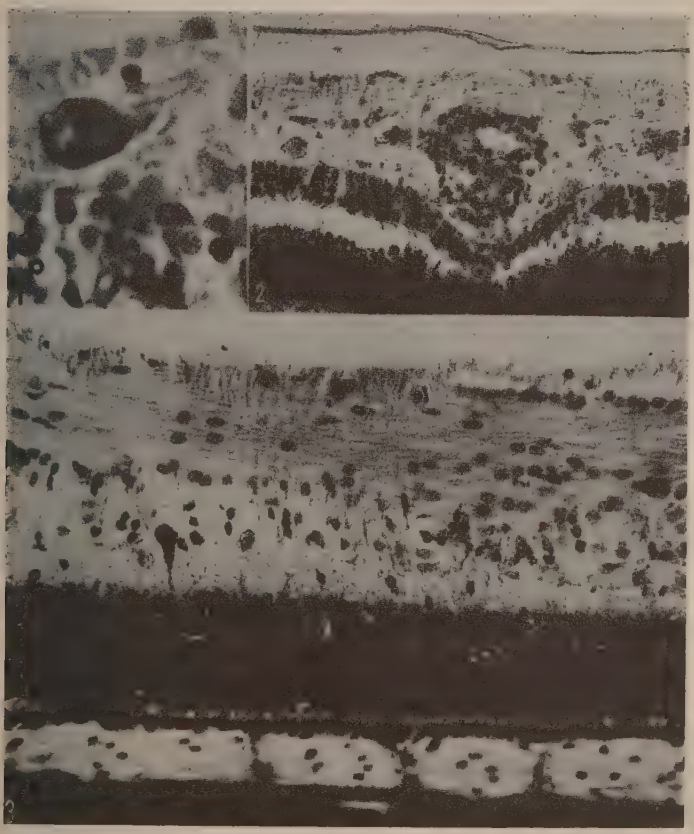

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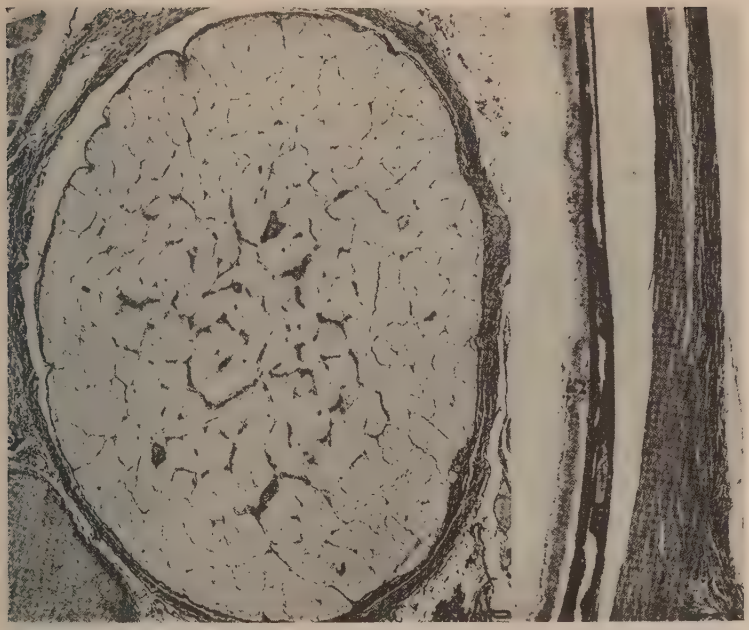

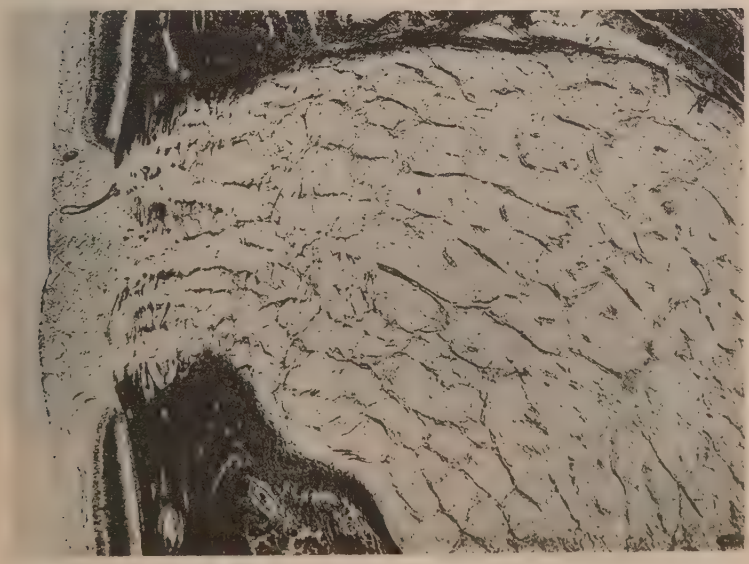

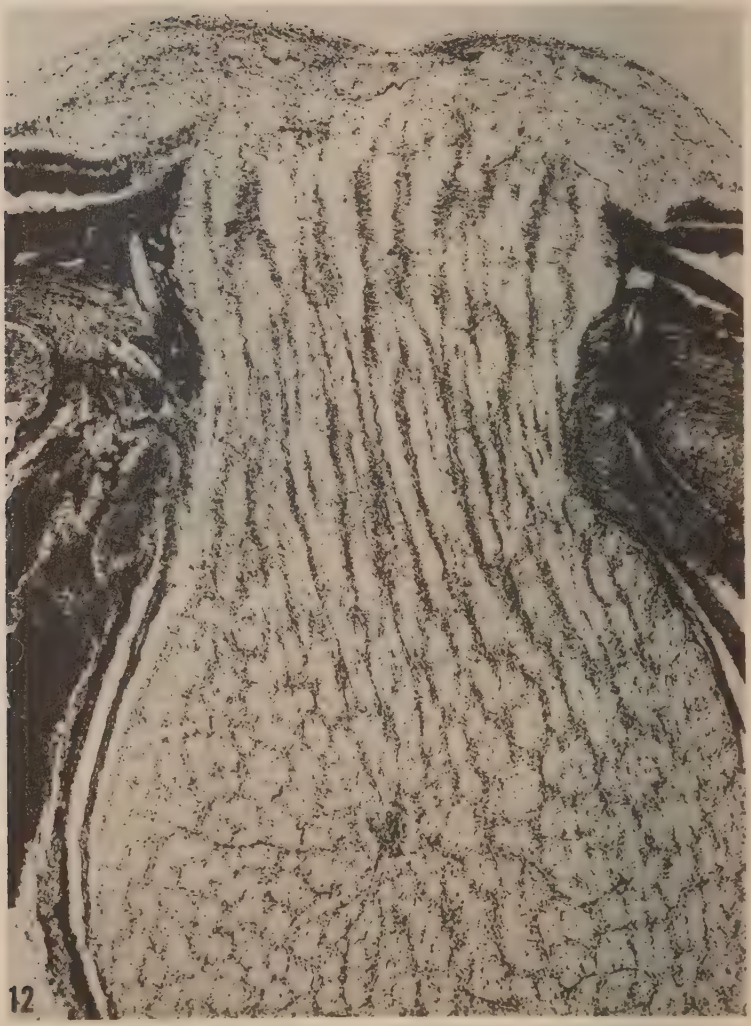
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LEGENDS TO ILLUSTRATIONS

Fig. 1. Ganglion cell in retina with swelling, margination of Nissl substance and peripheral migration of nucleus. This and all subsequent figures from sections stained with H. & E. × 600.

Fig. 2. Acute retinitis with perivascular cuffing and oedema which has displaced the internal limiting membrane. × 250.

Fig. 3. Atrophy of outer limbs of the layer of rods and cones and pyknosis of nuclei in this layer, as well as atrophy of the inner reticular layer. × 450. Key: a: optic fibre layer; b: ganglion cell layer; c: inner reticular layer; d: inner nuclear layer; e: outer reticular layer; f: outer nuclear layer; g: layer of rods and cones; h: pigment epithelium; i: tapetum; j: choroid.

Fig. 4. Intranuclear inclusion body in a cell of the inner nuclear layer of the retina. The arrow points to another inclusion body, in the inner reticular layer, out of focus in this plane. 20μ celloidin section, × 1500.

Figs. 5 and 7. Intranuclear inclusion bodies in glial cells in the optic nerve layer of the retina. × 1500.

Fig. 6. Intranuclear inclusion body in a glial cell in the inner reticular layer of the retina, same field as figure 4. The arrow points to the cell which was out of focus in figure 4. × 1500.

Fig. 8. Acute retinitis, perivascular cuffing, oedema and focal disorganization of the retinal layers in a freshly-fixed eye, celloidin embedded. × 220.

Fig. 9. Optic nerve showing widespread destruction of myelin without appreciable gliosis. × 40.

Fig. 10. Optic nerve with destruction of myelin and glial proliferation. × 50.

Fig. 11. Retina showing abrupt transition from normal architecture to loss of layer organization in a focal area of atrophy. Note invasion by clumps of black pigment and thinning of choroid underlying the atrophic retinal area. (The detachment of the choroid from the sclera is an artefact). × 30.

Fig. 12. Chronic optic neuritis. The extensive glial scarring apparently followed an acute optic neuritis in which papilloedema was present. × 50.

THE OCCURRENCE OF VACUOLATED NEURONES AND VASCULAR LESIONS IN THE MEDULLAS OF APPARENTLY HEALTHY SHEEP

By

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INTRODUCTION

In 1898 Besnoit and Morel reported the occurrence of vacuolated neurones in the medullas of sheep affected with scrapie. These observations were soon confirmed by Cassirer (1898) and later by Bertrand, Carré and Lucam (1937).

Brownlee (1940) after an extensive study of organs from scrapie sheep, came to the conclusion that vacuolation of neurones in the medulla and cord can be considered the only constant and specific lesion in scrapie. The same writer reported the occasional occurrence of vacuoles in sheep affected with other conditions, mainly louping-ill. However, in these conditions, vacuoles were of a much smaller size and could be seen in necrotic cells only, whereas in scrapie vacuoles appeared in completely normal cells. Similar observations were made by Holman and Pattison (1943) who found vacuolation of neurones in all clinical cases of scrapie, very occasionally in sheep suffering from other conditions,and in 1 only out of 59 apparently normal sheep (1.7 per cent). Finally, Wilson, Anderson and Smith (1950) found vacuoles in the neurones of the medulla in both natural and experimental scrapie.

Vascular lesions in scrapie have not been described by the majority of workers. Holman and Pattison (1943), in their study of 75 clinical cases of scrapie, 39 other than scrapie cases and 59 apparently healthy sheep, reported perivascular cuffings in one animal suffering from fits. Wilson, Anderson and Smith (1950) did however, observe perivascular infiltrations of lymphocytes in experimental scrapie. According to these writers, although perivascular lesions had a wide distribution in the C.N.S., only a small proportion of the vessels was so affected. The number of congregating lymphocytes was never great, and with few exceptions, did not completely encircle the affected blood vessel.

In view of the consensus of opinion that vacuoles in the neurones of the medulla form the only demonstrable microscopic lesion in scrapie, the laboratory diagnosis or rather the laboratory confirmation of a clinical diagnosis of scrapie was based on the finding of characteristic vacuolated neurones in the medulla. However, in the course of such routine histological examinations, the present writers observed vacuoles, identical with those seen in the brains of scrapie sheep, in the medullas of clinically healthy sheep. Further, vascular lesions, similar to these described by Wilson, Anderson and Smith (1950) in experimental scrapie were seen in the same medullas. In order to ascertain whether these findings were only a chance appearance, it was decided to examine the medullas from a considerable number of apparently scrapie-free sheep of various ages and breeds.

The present paper contains the observations made mainly on

sheep of the Cheviot breed: the findings in other breeds will be published later. In view of the very numerous histological sections examined in this work, only two types of lesions have been considered, vacuolation of neurones and perivascular infiltrations.

MATERIAL AND METHODS

The material for the present study consisted of medullas from 57 apparently healthy sheep. The animals were divided into three age groups as follows :—

(*a*) 20 Cheviot hoggs, about six months old. These animals came from flocks considered to be free of scrapie and were chosen at random from a consignment of six hundred and seventeen sheep sent to the Institute, very soon after their purchase. The sheep from each farm were in no sense selected, but they consisted of the whole or a large part of the draft of wether hoggs sold.

(*b*) 19 Cheviot hoggs, about 1 year old, obtained on two occasions from the Edinburgh abattoir. These animals were taken at random from very large consignments of sheep of the same breed and age.

(*c*) 18 sheep, over 3 years old, of the following breeds :—10 Cheviot ewes, 4 Border Leicester (cross-bred) ewes and 4 Blackface ewes, obtained on four separate days, in each case when considerable numbers of older sheep were slaughtered. None of these animals exhibited any clinical symptoms suggestive of scrapie.

Brains were removed immediately after slaughter and the medullas taken and fixed in 10 per cent formol-saline for two days. The medullas were then cut into three blocks as follows :—(1) extending from the posterior end of the pons to the anterior end of the olives, including the trapezoid body ; (2) cross section through both olives ; (3) between the posterior end of the olives and the medulla-cord junction.

The blocks were post-fixed in Bouin's solution for a further two days. After embedding in paraffin wax, serial sections 7μ in thickness were cut and stained with haematoxylin and eosin. Sixty consecutive sections of each block were examined microscopically, making a total of 180 sections examined from each medulla.

RESULTS

Vacuoles in neurones were observed in a large proportion of the medullas (73·7 per cent, with a variation of 61 per cent to 89 per cent.) The number of vacuoles found varied considerably from brain to brain, sometimes only one being found after a long search, while in other brains the vacuoles were fairly numerous, each section containing at least one. In a few brains the vacuoles were very numerous, as many as seven being seen in each microscopic field. The vacuoles were generally large causing ballooning of the cells, and were indistinguishable from those seen in brains from scrapie animals, either natural or experimentally produced (Figs. 1 to 7). Occasionally the vacuoles were found to contain eosinophilic round bodies (Fig. 7). The appearance of the cytoplasm and Nissl substance in the vacuolated neurones was unaltered but usually only a narrow rim remained around the vacuoles. By following vacuolated

cells in serial sections it was seen that the vacuoles were always confined to the cytoplasm. The nuclei although often displaced entirely to one side showed no degenerative changes (Figs. 6 & 7).

The majority of vacuoles were found in the caudal third of the medulla, between the posterior olives and the decussation of the pyramids, mostly in the dorsal motor vagus nucleus. Vacuoles were also seen in the anterior parts of the medulla and in other nuclei such as the inferior olive, the medial vestibular, paramedianus dorsalis, lateral reticular formation and facial. The incidence of vacuoles in the three age groups is given in Tables 1 to 3, there being a slight decline in frequency due to age; apart from this no difference was noticed between the vacuoles seen in young and old sheep.

Vascular lesions were also seen in the majority of animals examined. These changes were in the form of cellular infiltrations around blood vessels, the infiltrating cells being usually lymphocytes, although occasionally plasma cells were also present. Various degrees of perivascular infiltrations were observed ranging from slight accumulations of about two layers of cells at one side of a capillary (Figs. 8 & 11) to very severe, where several layers of cells completely surrounded blood vessels (Fig. 12). In a few cases the infiltrating cells formed nodules along the capillaries (Fig. 13), and in one case a large nodular infiltration in the wall of a small artery caused complete obliteration of the lumen (Fig. 9). Very frequently, apart from perivascular cuffings, focal infiltrations in the vicinity of large neurones could be seen (Fig. 10). Plasma cell infiltrations in the chorioid plexus were also of common occurrence. These vascular lesions were widely distributed and present in most sections of the affected medullas, but only a few vessels in each section were affected.

Tables 1 to 3 give details of the occurrence and severity of the vascular lesions. It can be seen that the total number of animals showing these changes varied in the three age groups from about 75 per cent in the 6 months old, to 94 per cent in the 1 year old and 83 per cent in the adult group. The severity of the lesions in the older sheep was greater than in the younger ones, 26·5 per cent of the affected brains of young sheep having severe lesions, while the corresponding figures for 1 year old sheep and adult sheep were 38·5 per cent and 53 per cent respectively.

A comparison of the results in respect of the occurrence of vacuoles in neurones and perivascular infiltration shows that in the majority of medullas both lesions were present, although in some cases perivascular lesions only were observed while in a few only vacuoles could be detected. This concurrent appearance of both types of lesions does not, however, necessarily mean that they are caused by a single factor, since the high incidence of both lesions makes this inevitable.

TABLE 1

HISTOLOGICAL LESIONS IN 6 MONTH OLD LAMBS

Serial No.	Vacuoles in neurones				Perivascular infiltrations				Remarks
		Frequency				Degree			
	Occurrence	Few	Fair nos.	Numerous	Occurrence	Slight	Moderate	Severe	
Exp. 1	+	+			+		+		
„ 2	+	+			+	+			Nodular infiltrations along capillaries.
„ 3	—				—				
„ 4	—				+		+		
„ 5	+		+		+	+			
„ 6	—				—				
„ 7	+	+			+		+		
„ 8	—				—				
„ 9	+	+			+	+			
„ 10	+			+	+			+	
„ 11	+			+	+			+	
„ 12	+	+			+		+		Focal infiltrations.
„ 13	+	+			+			+	
„ 14	+		+		+	+			
„ 15	+		+		+	+			
„ 16	—				+		+		Only medulla cord junction available for examination.
„ 17	+			+	+			+	Severe encephalitis with focal infiltrations and peri-arterial cuffings.
„ 18	+			+	+		+		
„ 19	+	+			—				
„ 20	—				—				
Total 20	14	7	3	4	15	5	6	4	

TABLE 2

HISTOLOGICAL LESIONS IN ONE YEAR OLD SHEEP

Serial No.	Vacuoles in neurones				Perivascular infiltrations				Remarks
		Frequency				Degree			
	Occurrence	Few	Fair nos.	Numerous	Occurrence	Slight	Moderate	Severe	
C. 72	+		+		+		+		Nodular infiltrations.
C. 73	—				+	+			
C. 74	+			+	+			+	Focal infiltrations.
C. 75	+	+			+		+		
C. 76	—				—				
C. 77	+	+			+			+	Focal infiltrations.
C. 78	+		+		+	+			
C. 79	+		+		+	+			
C. 80	+	+			+			+	
C. 81	+	+			+	+			
C. 82	+		+		+		+		
C. 83	+			+	+	+			
C. 84	+	+			+			+	
C. 85	+	+			+			+	
C. 86	+	+			+	+			
C. 87	+	+			+			+	
C. 88	+		+		+	+			Nodular infiltrations.
C. 89	+	+			+	+			
C. 90	+	+			+			+	
Total 19	17	10	5	2	18	8	3	7	

TABLE 3

HISTOLOGICAL LESIONS IN ADULT SHEEP

Serial No.	*Vacuoles in neurones*				*Perivascular infiltrations*				*Remarks*
	Occurrence	Frequency			Occurrence	Degree			
		Few	*Fair nos.*	*Numerous*		*Slight*	*Moderate*	*Severe*	
Scr. 1	+		+		+		+		
Scr. 14	+	+			+	+			
C. 56	—				—				
C. 57	+			+	—				
Scr. 23	—				+			+	
Scr. 24	—				+			+	
C. 52	+			+	+			+	Nodular infiltrations.
C. 53	+			+	+		+		Nodular infiltrations.
C. 54	—				+		+		
C. 55	+	+			+			+	
C. 97	+	+			+			+	
C. 98	+	+			+			+	
C. 99	+	+			+	+			Focal infiltrations of lymphocytes; and plasma cell infiltrations of choroid plexus.
C. 100	—				+	+			Nodular infiltrations.
C. 101	—				—				
C. 102	+		+		+			+	Plasma cell infiltration in choroid plexus.
C. 103	—				+			+	Multiple focal infiltrations — subacute encephalitis.
C. 104	+	+			+		+		Slight plasma cell infiltration in choroid plexus.
Total 18	11	6	2	3	15	3	4	8	

Scr. 1; Scr. 14; C. 56 & C. 57—Blackface Ewes.
Scr. 23; Scr. 24; C. 52 & C. 53—Border Leicester Cross Ewes.
C. 54 & C. 55; C. 97—104—Cheviot Ewes.

DISCUSSION

The significance of vacuolation of the neurones of the medullas from sheep as being an essential lesion of scrapie has been stressed by the majority of workers. It is in fact generally agreed that in view of their constant presence in the neurones of the medulla from scrapie sheep although admittedly, in some cases, they are very few in number, and in the absence of any other constant macroscopic or microscopic lesion, such vacuoles constitute the only pathological feature of diagnostic importance. The occurrence of vacuoles in non-scrapie animals was not considered by Brownlee (1940) of sufficient significance to reduce their importance for the diagnosis of scrapie, for according to him they were much smaller than those seen in scrapie, were only occasionally found, and when present were in necrotic nerve cells; whereas in scrapie the vacuolated neurones did not show any degenerative change. Holman and Pattison (1943) who examined a considerable number of medullas from both scrapie and non-scrapie sheep found vacuoles in all their 75 clinical cases of scrapie, whereas in 59 apparently healthy sheep they demonstrated vacuoles in one case only, and in 39 sheep dying from other diseases in four cases only. In spite of this insignificant incidence of vacuolated neurones in non-scrapie sheep, Holman and

Pattison were of the opinion that the finding of vacuoles in the medulla can only serve as additional proof of a tentative clinical diagnosis. The results of the present survey, showing a high incidence of vacuoles in apparently healthy sheep, would appear to suggest one of the following three possibilities, either that these sheep were in an incubation period of scrapie, or were suffering from a non-clinical form of the disease, or finally that vacuolation of the neurones is not directly connected with scrapie. One point however, seems to be certain that, at least, in sheep of the Cheviot breed, the finding of vacuoles in the medulla cannot be accepted as confirmatory evidence in the diagnosis of clinical scrapie, except perhaps if vacuolation is extremely severe.

Wilson, *et al.* (1950) reported that in the medullas from sheep experimentally infected with scrapie, vacuoles were always present, but that in most cases a more searching examination was usually required for their demonstration than in material from sheep affected with natural scrapie. Since similar considerations apply to the apparently healthy animals discussed in the present paper, and since vascular lesions identical with those described by Wilson, *et al.* (1950) in experimental scrapie were also observed in healthy sheep, it is possible that the lesions they observed in their experimental sheep were not due to the scrapie inoculum, but were, in fact, present in the medullas of the sheep at the time of inoculation.

CONCLUSIONS

Vacuoles in the neurones of the medullas of apparently healthy sheep identical or similar to those seen in scrapie animals are reported.

Perivascular cuffings in the medullas of apparently healthy sheep similar to those described in experimental scrapie were found in the majority of animals examined.

The significance of these lesions in the laboratory diagnosis of scrapie is discussed.

ACKNOWLEDGMENT

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Vacuolated neurones in the medulla from a natural case of scrapie × 190.

Fig. 2. Vacuolated neurones in the medulla from an experimental case of scrapie × 190.

Fig. 3. Vacuolated neurones in the medulla from an apparently healthy sheep × 190.

Fig. 4. Vacuolated neurone in the medulla from a natural case of scrapie × 485.

Fig. 5. Vacuolated neurone in the medulla from an experimental case of scrapie × 485.

Figs. 6 to 7. Vacuolated neurones in the medullas from apparently healthy sheep × 485.

Fig. 8. Slight perivascular infiltration in the medulla × 190.

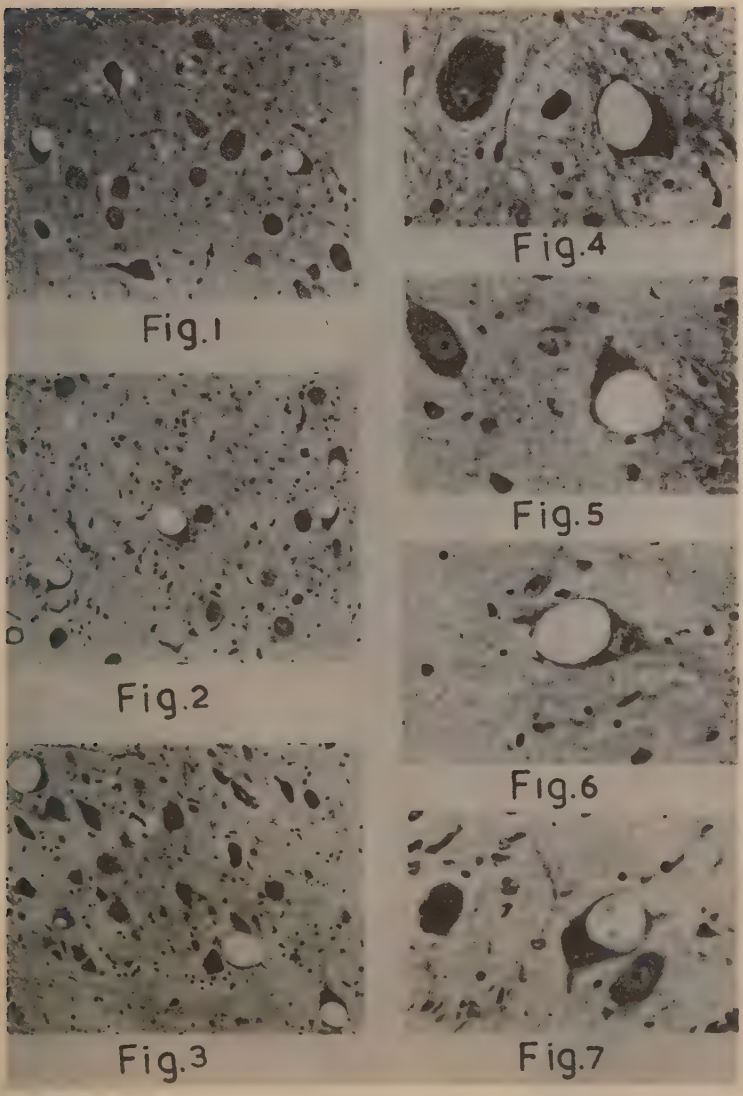
Fig. 9 Obliteration of a blood vessel due to a nodular infiltration within the lumen × 190.

Fig. 10. Focal infiltration in the vicinity of large neurones × 190.

Fig. 11. Moderate perivascular infiltration in the medulla × 485.

Fig. 12. Severe perivascular cuffing × 485.

Fig. 13. Nodular infiltration along the wall of a capillary × 485.



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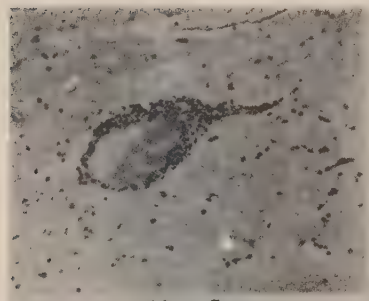
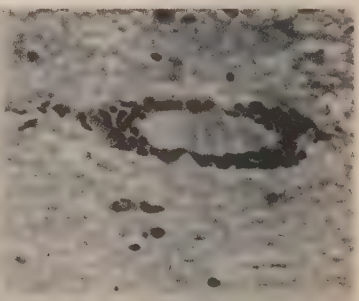


Fig.8



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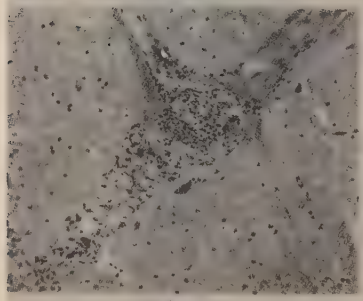

Fig.9

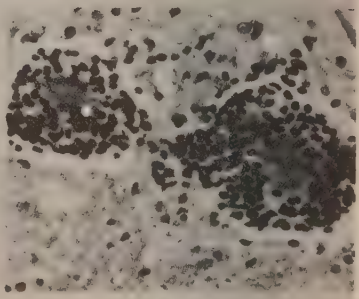

Fig.12

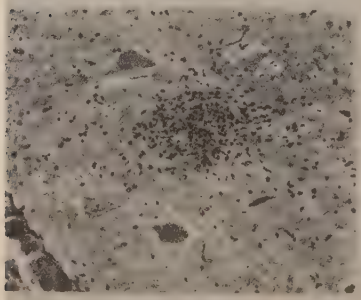

Fig.10

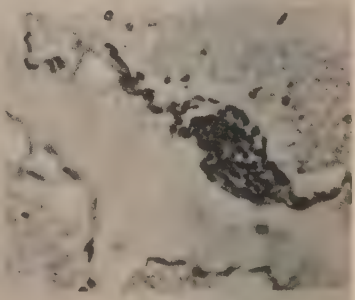

Fig.13

EXPERIMENTAL INFECTION WITH *MYCOBACTERIUM JOHNEI*

II. The Histopathology of Infection in Experimental Goats

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INTRODUCTION

The histopathology of Johne's disease in the intestines and mesenteric lymph nodes of cattle, sheep and goats has been described by a number of workers, including Twort and Ingram (1913), M'Fadyean (1918), Hallman and Witter (1933), McEwen (1939), Pande (1942), Levi (1948) and Stamp and Watt (1954). There are, however, very few descriptions of lesions in other situations. Mathews (1930) found numerous foci in the livers of clinically affected cattle, Borodenok (1941) reported lesions in the gall bladder, portal lymph node and spleen, and Levi (1948) described changes in many of the lymph nodes in goats in addition to the mesenteric group. In laboratory animals lesions have been described in the liver, spleen, pancreas and omentum (Hagan and Mansfield, 1930; Sahai, 1940; Francis, 1943; Lominski, Cameron and Roberts, 1956).

In the course of work in this Department on the relationship of the dietary status of goats and the size of the infecting dose to the development of infection with Johne's disease (Hirch and Lawrence, 1954; Hirch, 1956), a number of animals were slaughtered and their organs were available for histological examination. As the organs were also examined culturally for the presence of *M. johnei*, there was an excellent opportunity to correlate the histological lesions with the presence of bacilli, as it had previously been shown that *M. johnei* could be isolated from many of the organs of the body despite the absence of obvious lesions (Dunkin, 1928; Alexejeff-Goloff, 1929; Houthuis, 1932; Dunkin, Glover and Minett, 1941; Levi, 1948; Taylor, 1949, 1951, 1953).

METHODS

In the course of the experiments goats were infected by the following routes:—inhalation of aerosol spray, 2; intravenous injection, 9; intravenous injection and oral dosage, 6; oral dosage only, 26. There were also some animals which died within two months of the date of infection, or from intercurrent disease, but as no significant lesions were found in any of them they have not been included in this series of cases. Most of the animals were killed between 9 and 16 months after the date of infection.

As there was considerable variation in the number of organs examined histologically from individual animals, it was difficult in some cases to

compare the extent of the infection and the type of lesions present even in animals in the same experimental group.

Tissues were fixed in ten per cent. formol-saline, or formol-mercuric chloride saline, and embedded in paraffin wax. Sections were stained with haemalum and eosin to demonstrate the cellular changes. In order to determine the incidence of acid-fast organisms the Ziehl-Neelsen technique was used, with 1 per cent. toluidine blue as the counterstain, as it gave good tissue differentiation and also showed up clearly any non-acid-fast organisms which were present. Sections were also examined for the presence of *M. johnei* by the fluorescent method of Matthaei (1950) as modified by Gray (1953). This is considered in a separate paper, but the results are incorporated in Table 1, in which a positive sign in column 3 indicates the presence of organisms detected by either or both histological methods.

RESULTS

Lesions attributable to *M. johnei* were found to vary greatly from one animal to another, both in their distribution and in their histological appearances. It was not possible to ascribe these differences to any one factor and, though the route of infection and the time between infection and autopsy played some part, it is possible that differences in individual resistance and the use of a number of strains of *M. johnei* may have influenced the results. Taking the group as a whole, lesions were found in all the organs examined, as shown in Table 1.

Lungs

Almost all the sections examined showed some degree of collapse, associated with vesicular emphysema; in addition, many of the alveolar walls were thickened due to cellular infiltration with macrophages and lymphocytes. The capillaries in these areas were usually prominent, and a combination of collapse and infiltration led to the formation of a number of vascular nodules, (Fig. 6). There were also nodules which were completely avascular, formed purely from cellular infiltration or proliferation. A more severe change, also quite commonly seen, was the development of interstitial pneumonia, but the affected areas were small and irregularly distributed. Generally speaking, the fibrous tissue remained very cellular. Catarrhal bronchitis was not uncommon, but there was no definite increase in the peribronchial lymphoid tissue. In some of these lymphoid areas, however, there were small foci of macrophages. Similar nodules were present surrounding some of the smaller arteries.

The changes so far described cannot be regarded as being in any way specific, but in a number of animals definite lesions were present. These consisted of a discrete nodule of macrophages with pale or foamy cytoplasm, and in some cases the appearance was almost identical to the typical symplasma of the intestinal lesions (Fig. 5). In some animals there appeared to be no reaction to the presence

Goat No.	Lungs 1	Lungs 2	Lungs 3	Liver 1	Liver 2	Liver 3	Kidneys 1	Kidneys 2	Kidneys 3	Spleen 1	Spleen 2	Spleen 3	Intestine 1	Intestine 2	Intestine 3	Mesenteric L.N. 1	Mesenteric L.N. 2	Mesenteric L.N. 3	Hepatic L.N. 1	Hepatic L.N. 2	Hepatic L.N. 3	Other L.Ns. 1	Other L.Ns. 2	Other L.Ns. 3	Other organs 1	Other organs 2	Other organs 3
Intravenous only																											
1807	+	L	+	—	SL	—	+	SL	+	+	N	+	o	L	+	+	L	+	—	L	—						
1808	+	L	+	+	L	+	—	SL	+	—	N	—	o	o	o	+	SL	+	—	N	—						
1809	—	N	—	+	L	+	+	SL	+	—	N	—	o	o	o	+	N	—	o	o	o						
1811	o	SL	+	+	L	+	—	N	—	—	SL	+	o	L	+	+	L	+	—	N	—						
1812	o	SL	+	—	SL	+	—	N	—	—	N	—	—	N	—	+	L	+	—	SL	+						
1725	o	L	+	o	EL	+	o	N	—	o	N	—	o	SL	+	o	SL	+	o	L	+						
1728	o	L	+	o	SL	—	o	o	o	o	N	—	o	SL	+	o	N	—	o	o	o						
																						Bronchial					
1693	o	EL	+	o	EL	+	o	N	+	o	N	+	o	L	+	o	L	+	o	EL	+	o	L	+			
1705	o Lungworm		+	o	EL	+	o	o	o	o	o	o	o	EL	+	o	L	+	o	L	+						
Aerosol																											
BB945	o	L	+	o	N	—	o	N	—	o	o	o	o	o	o	o	N	—	o	o	o						
																						Retro-pharyngeal			Tonsil		
A4419	+	L	+	+	SL	+	o	o	o	+	N	+	—	N	+	+	SL	+	+	N	+	+	SL	+	—	N	—
Intravenous and oral																									Tonsil		
1644	+	L	+	+	L	+	+	N	+	—	o	o	o	EL	+	+	EL	+	—	L	+				—	o	o
1648	—*	N	+	—*	L	+	—	N	—	+	L	+	o	EL	+	+	EL	+	+	L	+						
																						Retro-pharyngeal			Tonsil		
1650	+	L	+	+	L	+	+	L	+	+	SL	+	o	o	o	+	L	+	+	L	—	+	L	+	+	o	o
																						Retro.					
1665	+	L	+	+	L	+	—	L	+	—	N	+	o	o	o	+	EL	+	+	EL	+	+	N	+			
																						Retro.					
1667	—	N	—	+	SL	+	+	L	+	+	N	+	o	o	o	+	EL	+	+	EL	+	+	L	+			
																						Retro.			Tonsil		
1669	+	SL	+	—	L	+	+	L	+	+	N	+	o	EL	+	+	EL	+	+	EL	+	+	o	o	+	o	o
Oral only																						Retro.			Tonsil		
1896	+	o	o	+	o	o	+	o	o	+	o	o	o	EL	+	+	EL	+	+	EL	+	+	L	+	+	L	+
1895	—	N	—	—	N	—	—	N	—	—	N	—	+	SL	+	+	SL	+	—	N	—						
																									Pancreas		
1	—	N	—	—	N	—	—	N	—	—	N	—	+	N	—	+	SL	+	—	o	o				o	N	—
3	o	N	—	—	N	—	o	N	—	o	N	—	—	SL	+	o	SL	+	o	o	o						
3B	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—						
4	+	L	+	+	L	+	o	N	—	+	N	—	+	EL	+	+	EL	+	+	L	+						
																									Uterus		
2	—	N	—	—	SL	—	+	SL	+	—	N	—	+	L	+	+	L	+	+	SL	+				+	N	+

Goat No.	Lungs 1	2	3	Liver 1	2	3	Kidneys 1	2	3	Spleen 1	2	3	Intestine 1	2	3	Mesenteric L.N. 1	2	3	Hepatic L.N. 1	2	3	Other L.N.s 1	2	3	Other organs 1	2	3
4B	+	SL	+	—	L	+	o	N	—	+	N	+	+	L	+	+	EL	+	+	EL	+				Uterus +	N	—
																									Ovary +	N	+
1940	—	o	o	+	o	o	o	o	o	o	N	+	o	o	o	+	L	+	o	o	o	+	o	o	Tonsil +	SL	+
1959	+	L	+	+	L	+	+	N	+	+	N	+	+	EL	+	—	EL	+	+	L	+				Uterus +	N	+
																									Ovary +	N	+
1961	—	N	—	+	SL	+	—	SL	+	—	N	—	—	N	—	+	L	+	—	N	—							
1963	+	SL	+	+	L	+	—	SL	+	+	N	+	+	L	+	+	L	+	o	SL	+							
1965	—	N	—	—	N	—	—	N	—	—	N	—	+	N	+	+	N	—	N	o	o							
1966	+	L	+	+	L	+	+	N	+	+	SL	+	+	EL	+	+	EL	+	+	o	o							
																									Uterus		
1967	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—				—	N	—
1968	—	N	—	—	SL	+	—	N	—	+	N	+	—	N	+	—	SL	+	o	o	o							
1969	+	L	+	+	SL	+	+	N	—	+	N	—	+	N	—	+	L	+	o	o	o							
																									Uterus		
1962	—	L	+	—	L	+	—	N	—	—	SL	+	+	EL	+	+	EL	+	+	SL	+				—	SL	+
6	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	+	N	—	—	N	—							
7	—	N	—	—	L	+	—	N	—	—	N	—	—	L	+	—	L	+	—	SL	—							
																									Uterus		
9	—	N	—	+	L	+	—	N	—	+	N	—	+	L	+	+	L	+	+	L	+				+	N	+
																									Pancreas		
1960	+	L	+	+	L	+	+	SL	+	+	SL	+	+	EL	+	+	EL	+	+	L	+				—	L	—
																									Pancreas		
1954	+	L	+	+	L	+	—	SL	—	—	N	—	+	EL	+	—	EL	+	o	L	+				o	N	—
																									Pancreas		
1964	—	L	+	—	L	+	—	N	—	—	N	—	+	EL	+	+	L	+	o	o	o				o	N	—
8	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	—	N	—	o	o	o							
5	—	L	+	—	L	+	—	SL	+	—	N	+	+	EL	+	+	L	+	—	L	+							

* Cultures negative, but organisms seen in material prepared for culture.

1=Cultures. *2*=Lesions. *3*=Presence of organisms in section. o=not examined. +=organisms present. —=no organisms detected.
SL=slight lesions. L=average lesions. EL=extensive or severe lesions. N=no lesions. LN=lymph node.

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TABLE 2

THE DISTRIBUTION AND SEVERITY OF HISTOLOGICAL LESIONS IN RELATION TO THE ROUTE OF INFECTION

Route of infection and number of animals in each group.	*Lungs*			*Liver*			*Kidneys*			*Spleen*			*Intestine*			*Mesenteric L.N.*			*Hepatic L.N.*		
	A	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*	*A*	*B*	*C*
Oral and intravenous. 6 animals.	3	1	2	4	2	0	4	0	2	1	1	2	3	0	0	6	0	0	6	0	0
Intravenous. .. .. .. 9 animals.	5	2	1	6	3	0	0	3	4	0	1	7	4	2	1	5	2	2	4	1	2
Oral. .. .. 26 animals.	9	2	13	12	4	8	0	6	18	0	3	22	14	2	9	17	4	5	8	4	5
Aerosol. .. .. 2 animals.	0	2	0	0	1	1	0	0	1	0	0	1	0	0	1	0	1	1	0	0	1

A=Marked lesions. *B*=Slight lesions. *C*=No lesions.
The oral and intravenous group received part of the infecting doses as emulsified scrapings of infected intestine.
The other three groups received fluid cultures of *M. johnei*.

of the foci of macrophages, but in others there was a definite perinodular lymphocytic infiltration. The nodules occurred in any part of the lung: in some instances they appeared to be related to arteries, and in one animal they occurred in the inter-lobular lymphatics. None were observed peribronchially. In one animal only (1693) lesions had reached macroscopic size, with numerous nodules up to about 2 mm. in diameter, and larger areas formed by the confluence of several nodules. Lesions did not appear to be related to the bronchi. Histologically some nodules had a cellular centre, consisting of macrophages, plasma cells, neutrophils and a few eosinophils (Figs. 3 and 4); in others the material was caseous and there was some evidence of commencing calcification, (Fig. 1). Surrounding the central area was a very pale staining zone, consisting mainly of macrophages with vacuolated cytoplasm and indistinct cell boundaries, and a small proportion of fibroblasts which tended to be arranged in a radial fashion. A small number of giant cells were present, several of them being clearly of the Langhans type (Fig. 2). Immediately outside the pale zone was a very cellular tissue composed of closely packed lymphocytes, plasma cells and macrophages, with a small number of neutrophils in a few areas. There were also foci of fibroblastic proliferation. The cellular zone varied markedly in thickness and merged quite abruptly at its periphery into comparatively normal lung. Another lesion, seen only in this animal, was a periarteritis, involving a number of the pulmonary arteries, in which the adventitia had become greatly thickened by the deposition of collagen fibres in the form of loose concentric whorls. This reaction was not however considered to be specifically related to the infection with *M. johnei*.

Lesions occurred in animals infected by any route, good examples being found in animals infected orally, though goat 1693 was infected by intravenous injection only. Organisms were recovered from the lungs by cultural methods in a large proportion of animals, but always proved difficult to detect in sections as they were present in very small numbers, and usually occurred singly or in pairs. In several cases reddish-purple granules occurred in the cytoplasm of the macrophages, and were also present in the caseous areas of goat 1693. These may well represent degenerating or involution forms of *M. johnei* (Borodenok, 1941; Stamp and Watt, 1954).

Liver

None of the livers examined showed any macroscopic changes, even where subsequent histological examination showed extensive lesions. In the majority of cases the hepatic cells in general appeared normal, though where numerous lesions were present cloudy swelling was often observed (Fig. 7).

Two varieties of lesion were observed, the commoner of which (Type 1) consisted of an almost circular focus of macrophages, intermingled with a varying proportion of lymphocytes. Though

these cells occurred anywhere within the nodule they tended to be most numerous at the periphery, in some cases forming a definite zone. The nodules were always avascular. In many the macrophages had lost their distinct cell outlines, giving rise to the formation of small areas of symplasma. In some cases the centre of the nodule consisted of one or more multinucleate giant cells, resembling the lesions described in cattle by Mathews (1930). The lesions were usually separated from the liver tissue round the greater part of their periphery by a narrow cleft, often containing blood and in a few cases definitely continuous with a sinusoid (Figs. 9 and 10). In one animal a swollen Kupffer cell still lying in its sinusoid was observed to contain several acid-fast bacilli, and from observations made in mice (Harding, unpublished) it has been possible to follow the development of the lesions from the primary infection of the Kupffer cell up to the fully-formed nodule described above. It appears that as the lesion increases in size by the multiplication or infiltration of macrophages the adjacent liver cells become displaced, but the sinusoidal endothelium is not disrupted and the continuity of the sinusoid is not necessarily lost. This view is also held by Francis (1943). Similar lesions were also found in mice by Lominski, Cameron and Roberts (1956).

Type II lesions appeared to be more active. The nodules were frequently vascular, or surrounded by a vascular reaction, and their boundaries were relatively ill defined, as inflammatory cells extended for some way along the sinusoids and between the hepatic cells round the periphery (Fig. 11). Active hepatic cell destruction appeared to be taking place (Fig. 12).

It was not uncommon to find lesions of more than one type in the same animal. The lesions were present in any part of the lobule, but were more prevalent in the outer zone (64 per cent.) than in the intermediate zone (28 per cent) or the inner zone, (8 per cent). In some animals lesions were also commonly found sub-capsularly. In most cases lesions were also present in the portal tracts (Fig. 8). The predominating cells were again macrophages, but lymphocytes tended to be more numerous than in the nodular lesions. In many of the portal lesions there was also a diffuse fibroblastic proliferation, but this never tended to produce encapsulation. In one animal the portal reaction was particularly marked, and large areas of symplasma were present, the central regions suggesting the onset of cell necrosis.

Organisms were never very numerous in the liver; they were most readily detected in the portal lesions, but they were also visible in some of the typical nodules. Apart from the exceptions mentioned, organisms were never observed outside the lesions. The numbers varied appreciably from one animal to another, and did not appear to be related either to the route of infection or its duration. In one animal which had been infected about six months previously a monocyte present in a small branch of the portal vein

was found to contain a small group of typical acid-fast bacilli.

Spleen

In general, no specific lesions were demonstrable, though some degree of follicular hyperplasia, and a diffuse fibrosis of the pulp was not uncommon. Some sections showed a definite increase in the number of endothelial cells, in one case to such a degree that the majority of sinusoids were obliterated. This was the only case in which organisms were seen in the Ziehl-Neelsen section; they were scattered throughout the section in ones and twos, the total number present being small.

Kidney

The kidney parenchyma sometimes showed cloudy swelling of the tubular epithelium. Increased cellularity and swelling of the glomerular tufts was also observed. Specific lesions were present in 13 cases and varied markedly in degree from one animal to another. In nine animals lesions were most numerous in the cortex, whereas in the other four they were observed only in the medulla. The changes were essentially those of an interstitial nephritis, the infiltrating cells being predominantly lymphocytes and macrophages. The lesions varied in size from small groups of cells causing only a slight separation of the tubules up to areas involving about a third of the thickness of the cortex, within which all the normal kidney tissue had been wholly or partially destroyed (Fig. 13). It was observed that the basement membrane of the tubules appeared to disappear first, followed by separation of the epithelial cells and loss of the tubular structure. The epithelial cells, however, appeared to persist for some time and became incorporated in the inflammatory tissue. There was never any evidence of vascular reaction round the lesions. The largest lesions occurred in animals infected by the intravenous route, but three of the cases showing only medullary lesions (Fig. 14) had been infected orally.

Typical acid-fast organisms could be demonstrated in small numbers in most of the lesions which contained macrophages, but were never observed in the normal kidney tissue nor in areas of interstitial nephritis in which macrophages were not present.

Intestine

Sections of intestine were not available from seven of the animals, and in a number of others only one or two pieces of tissue had been taken from each case and even then not from corresponding sites. Later in the series at least three blocks of tissue were taken, one each from duodenum, jejunum and ileum, with additional tissue from any site where it was considered necessary.

The histology of the intestinal lesions of Johne's disease have been described in cattle by M'Fadyean (1918) and Hallman and Witter (1933), in sheep by McEwen (1939) and Stamp and Watt (1954), and in goats by Pande (1942) and Levi (1948).

In general the histogenesis of the lesions appears to be very

similar in all species, commencing as focal aggregations of macrophages (epithelioid cells) in the lamina propria, followed by a progressive increase in size of the lesions and the disappearance of the normal tissues. A number of different types could be recognised in the further development of the lesions in this series of goats, which corresponded quite closely to the lesions found in the naturally occurring disease in sheep (Stamp and Watt, 1954).

In some cases the lesions, however extensive, consisted almost entirely of macrophages. Sometimes the reaction appeared to be limited by the muscularis mucosae, but in other cases it had extended into the submucous tissues, with varying degrees of disappearance of the muscularis mucosae. Intestinal villi were distended or occasionally completely replaced by the inflammatory tissue, but little actual thickening of the mucosa appeared to have occurred. This is the type of lesion described by Pande (1942) as occurring in naturally infected goats, and is similar to Group I of Stamp and Watt.

A more active variety of this type of lesion was more common. The macrophages were mixed with lymphocytes and plasma cells; in some cases they were outnumbered by them. In a very small number of cases Langhans giant cells were present, both in the mucosal and submucosal lesions. The foci almost always spread beyond the muscularis mucosae, and quite often involved the intestinal musculature and the serosal surface. The peritoneal surface showed a diffuse lymphocytic reaction, and there were also marked focal lesions involving the lymphatic vessels in which the principal cell type was a vacuolated macrophage. The lesions appeared to start as a lymphangitis with a subsequent peri-lymphatic reaction (Fig. 16). It was observed that cells within the lumen of the lymphatic often showed necrotic changes, and in some cases caseous foci were present.

In a small number of animals the early foci of macrophages tended to remain discrete and appeared to be surrounded by a definite lymphocytic zone. The lesions remained small and involvement of the submucous zone was never observed. This type is rather similar to Group 2 of Stamp and Watt (1954).

Necrosis and caseation of the lesions in the mucosa and the submucosa was a prominent feature in a few cases. The caseous tissue showed small areas of early calcification, and neutrophils were quite numerous in the surrounding tissue reaction. In one case fibroblasts were in the process of encapsulating the lesions. As *M. johnei* was present in very large numbers in one of the submucosal lesions, and no other micro-organisms could be observed, it was considered that a neutrophil response must be part of the defence mechanism in certain cases. In general, however, it was observed that where neutrophils were prominent it was very difficult to show the presence of *M. johnei* microscopically. Hallman and Witter (1933) observed that neutrophils occurred in a small proportion of

intestinal lesions in cattle. In one animal in which caseation was present in the depth of the mucous membrane and in the submucosal lesions there was also extensive necrosis of the surface layers of the mucosa and a fibrinous exudate was present. Degenerating material in the form of intensely basophilic strands and bizarre shapes extended for some distance into the deeper layers of the mucosa and could be seen between the glands. Enormous numbers of *M. johnei* were present in the necrotic material. This type of reaction most nearly corresponds to Group 4 of Stamp and Watt (1954), though the reaction in the goat appears to be more pronounced and there is also some variation in the numbers of *M. johnei* present in the lesions.

The numbers of organisms present in the lesions of all types varied widely. In general they were easiest to find in the lesions corresponding to Groups 1 and 4, and hardest in Group 2. The greatest variation occurred in the sub-type of Group 1. They tended to be most numerous in the mucosal lesions, less numerous in the submucosal foci, and only rarely visible in the peritoneal and lymphatic lesions, though exceptions to this general rule occurred. In some animals in which the histological changes were typical, faintly acid-fast coccoid granules were present in the cytoplasm of some of the macrophages and appeared to be similar to those described by Stamp and Watt (1954).

There was no apparent correlation between the duration of infection and the severity of the lesions, and little or no difference between the various routes of infection.

Lymph nodes

Mesenteric nodes were available from all the animals, hepatic nodes from 29 and other nodes from seven.

Macroscopically affected nodes were enlarged and oedematous, considerable amounts of milky fluid escaping when they were cut. In some cases there was a very marked lymphangitis, caseous nodules occurring along the course of some of the lymphatic vessels, associated with varying degrees of caseation, calcification and fibrosis of the lymph nodes. These advanced changes were confined to the mesenteric nodes apart from two animals in which small caseous areas occurred in the hepatic lymph nodes also. Similar changes have been recorded by numerous authors in sheep and goats, including M'Fadyean and Sheather (1916); Holmboe and Slagsvold (1934); Pande (1942); Levi (1948) and Stamp and Watt (1954).

Histologically the lesions observed were in general similar to those described by M'Fadyean (1918), Hallman and Witter (1933), McEwen (1939), Pande (1942), Levi (1948) and Stamp and Watt (1954). They first appeared in the cortex, often only in a limited sector, as focal aggregations of macrophages. These most usually formed clearly defined nodules, up to about 15 cells being visible on

section, with abundant, pale, non-granular cytoplasm which showed a marked tendency towards symplasma formation. Sometimes the macrophages were mixed with a proportion of lymphocytes, but plasma cells were never observed in relation to the lesions, though this feature was described in cattle by M'Fadyean (1918). The "cell groups" occurred most commonly in the lymphoid tissue, but occasionally in the germinal centres (Fig. 18). Similar lesions were seen in the subcapsular sinus in two cases, though generally the macrophage reaction in this site was more diffuse. By increase in size and coalescence of the primary foci large areas of symplasma often developed leading to obliteration of the normal cortical structure, the germinal centres appearing to be most resistant to the process. Giant cells were present in three cases (Fig. 17), but could not be regarded as a usual feature, though in other species they seem to occur frequently (M'Fadyean, 1918). In a few sections a diffuse fibroblastic reaction had occurred, so that areas of cortex consisted of a cellular connective tissue. M'Fadyean (1918) considered that this tissue was actually formed by the macrophages, but in the goats the tissue, though cellular in character, was shown to contain mature collagen fibres. Whilst both caseation and calcification had occurred in a number of cases on a normal diet, no calcification was observed in any of the animals which had been fed a diet deficient in calcium. The caseous tissue was encapsulated to varying degrees, the reaction being most marked round calcified areas.

The medullary changes were relatively non-specific in character and consisted of distension of the sinusoids with fluid and macrophages. The cells remained discrete and no attempt at symplasma formation was observed. In general, medullary lesions only occurred in nodes in which the cortex showed extensive involvement. The afferent lymphatic vessels in the capsule frequently showed dilatation and contained numerous macrophages, some of which were undergoing necrosis. Efferent lymphatics were less frequently affected, and to a milder degree.

Lesions were observed in the visceral and skeletal lymph nodes from all parts of the body and also in the tonsils, but in most cases these had not progressed beyond the formation of cortical foci of macrophages.

In conformity with the findings of Eveleth and Eveleth (1943), *M. johnei* was generally more easily detected in the lymph node lesions than in the organs which drained into them, and in some cases in which organisms could not be detected in intestinal lesions they were present in large numbers in the nodes. Occasionally, however, extensive typical lesions were observed in the nodes although organisms could not be demonstrated in them either culturally or histologically, but in a few cases the pinkish, granular forms already described were observed. Organisms were apparently always intracellular except where caseous material was present.

Even when present in the cortex in extremely large numbers they were either very hard to demonstrate or apparently completely absent from the medulla, and when they did occur were present only as single organisms or in very small groups in individual macrophages.

Uterus

Sections of the uterus were examined from six animals, one of which (1967) showed no evidence of having become infected with *M. johnei*, as no lesions were detected in any of the organs and cultures were uniformly negative. Of the other five, positive cultures were obtained from the uterus in four cases, and in four of them also small numbers of *M. johnei* were observed histologically in the sub-epithelial connective tissue. Slight lesions were distinguished in one. In the two ovaries examined organisms were present, but lesions were not apparent.

Pancreas

Lesions were observed in one animal out of four examined. They were similar in character to the nodules found in the liver (Fig. 15).

DISCUSSION

The views of M'Fadyean (1918) that Johne's disease is strictly limited to the intestine and mesenteric lymph nodes prevailed for many years, despite the claim of Alexejeff-Goloff (1929) to have isolated the organisms from many sites in cattle suffering from the advanced clinical disease. Earlier reports of lesions probably involving the bladder (Bergman, 1913), and of necrotic foci in the liver and mesenteric lymph nodes of a gnu (Jarmai, 1922), were quoted by Alexejeff-Goloff. In more recent years there have been a number of papers recording the isolation of *M. johnei* from practically all the organs and lymph nodes in the body (Houthuis, 1932; Borodenok, 1941; Dunkin, Glover and Minett, 1941; Taylor, 1949, 1951, 1953; Doyle, 1954). Histological lesions in the liver and gall bladder in cattle were recorded by Mathews (1930) and Borodenok (1941), and in various sites in laboratory animals by Hagan and Mansfield (1930), Sahai (1940), Francis (1943) and Lominski, Cameron and Roberts (1956).

It appears from the present series of goats that histological lesions associated with the presence of *M. johnei* may be as widespread as the distribution of the organism throughout the body, and a limited examination of organs from two clinical cases in cattle revealed similar lesions in this species (Harding, unpublished). Thus the conception of the disease as a localised invasion of the intestine and mesenteric lymph nodes, with only sporadic bacteraemic episodes in the advanced clinical stages, would not appear to be correct. Haematogenous spread must commence comparatively early in the disease process, but the organisms do not

appear to multiply to any extent in what may be regarded as natural host species or give rise to macroscopic lesions, except in the intestine and lymph nodes, although one exception has been quoted in this paper (goat 1693) and one noted by Jarmai (1922). Since Sahai (1940) has shown that the intra-peritoneal injection of dead *M. johnei* into guinea-pigs can give rise to lesions identical with those caused by living organisms, the question of the survival of *M. johnei* in the "unusual" organs must remain an open one. There was close agreement throughout this series between the presence of histological lesions and the ability to culture the organisms (Table 1), but this cannot be regarded as proof that the organisms are surviving since there may well be a continuous low grade bacteraemia with ingestion of organisms by the fixed tissue histiocytes. In support of this view it was found that in the lungs organisms could often be demonstrated microscopically in individual macrophages lying in the alveolar walls more readily than in the lesions.

There are two obvious routes for the dissemination of infection from the intestine. Firstly there is progressive lymphatic spread, organisms eventually reaching the blood stream through the thoracic duct; and secondly there is direct invasion of the portal venous circulation. That the latter can occur has been shown by the demonstration of an infected macrophage in an intra-hepatic branch of the portal vein: the distribution of lesions within the liver lobules also suggests this route of spread. The presence of some lesions abutting on the central veins indicates that infection probably passes through the liver and enters the systemic circulation. In mice extensive lesions occur in the liver and spleen (Francis, 1943; Harding, unpublished), and their distribution also points to portal spread. As lesions actually involving the walls of the veins have not been observed, extensive lesions in the portal tracts probably arise by lymphatic spread from the intralobular lesions, or possibly by systemic spread through the hepatic artery. Taylor (1953) isolated *M. johnei* from five out of 12 livers in animals infected experimentally and killed before reaching the clinical stage of the disease, and from ten out of ten livers in animals killed because of the advanced natural disease. In this series liver sections were examined from 41 goats; 23 showed marked lesions, nine slight lesions, and nine no lesions, but of these nine negative animals five had no lesions anywhere, and four showed complete or incomplete primary complexes only, with no evidence of further progression. It is a noteworthy feature that even in cases where liver lesions were numerous the organ showed no macroscopic changes, and Francis (1943) also emphasises this feature in mice. Nevertheless, Jarmai (1922) has shown that liver lesions can become macroscopic, and there is the possibility that lesions in one of our goats might have become caseous if the disease had been allowed to run a longer course.

Although lung lesions do not appear to have been described

previously, specific lesions were observed in over 70 per cent of the lungs of animals in which the infection had become established. As only one or two blocks were taken from each set of lungs it is probable that in fact lesions were present in an even higher proportion of cases. The smaller lesions bore some resemblance to the early stages of *M. tuberculosis* infection, and the advanced lesions observed in goat 1693 had a cellular composition similar to that found in avian tuberculosis in the lung of the pig (Feldman, 1938). The recovery of *M. johnei* in culture from the lungs showed very close agreement with the histological findings, and in no case was *M. tuberculosis* isolated, although unfortunately no cultures were made from goat 1693. This has been taken as indicating that the lesions are attributable to *M. johnei* infection, although as was also observed in the liver, it was often not possible to detect acid-fast bacilli in the actual lesions. On the other hand, although toluidine blue was used as the counterstain and Gram-stained sections were examined in many cases, no other micro-organisms were ever detected within the lesions. A small number of goats developed lungworm infection, but where there was any doubt as to the nature of the lesions the sections were omitted from the series.

The presence of *M. johnei* immediately below the epithelium in four non-pregnant uteri suggests a possible source of the infection found in a six weeks old bovine foetus by Lawrence (1956), for if organisms are present in a similar situation in the bovine uterus they could be exposed by the denudation of the uterine caruncles and thus gain access to the lumen of the uterus and the foetus.

A comparison of the lesions as between the different routes of infection failed to reveal any significant differences between them (Table 2). The variations within a group were as great as the variations between groups, and in one of the experiments where the size of the infecting dose was the only variable, it was observed that some of the animals developing the most marked lesions had received the smallest dose. It would thus appear that in the development of the disease the susceptibility of the individual animals was more important than any of the other variables.

CONCLUSIONS

The histopathology of Johne's disease in experimentally infected goats has been described. Lesions were found in all the lymph nodes and organs examined. The mesenteric lymph nodes and the intestines showed lesions most frequently, but they were found in all the livers and 70 per cent of the lungs in animals in which the infection had progressed beyond the primary complex. Other organs and nodes were inconstantly involved, the hepatic lymph nodes, kidneys and spleen, in that order, being most likely to show lesions.

There was a high degree of correlation between the presence of

lesions and the isolation of *M. johnei* in culture. Some of the discrepancies may have been due to the localised distribution of lesions within an organ; insufficiently sensitive techniques probably account for others. There is also the probability that in some instances organisms had only just reached the organ and had not had time to give rise to histologically recognisable changes; or on the other hand that lesions persisted for some time after the organisms had been killed by the body defences.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Large lesion, lung. (goat 1693). H. & E. × 36.
Fig. 2. Giant cells at margin of caseous centre of Fig. 1. H. & E. × 345.
Fig. 3 and 4. Lung lesions. (goat 1693). H. & E. × 76.
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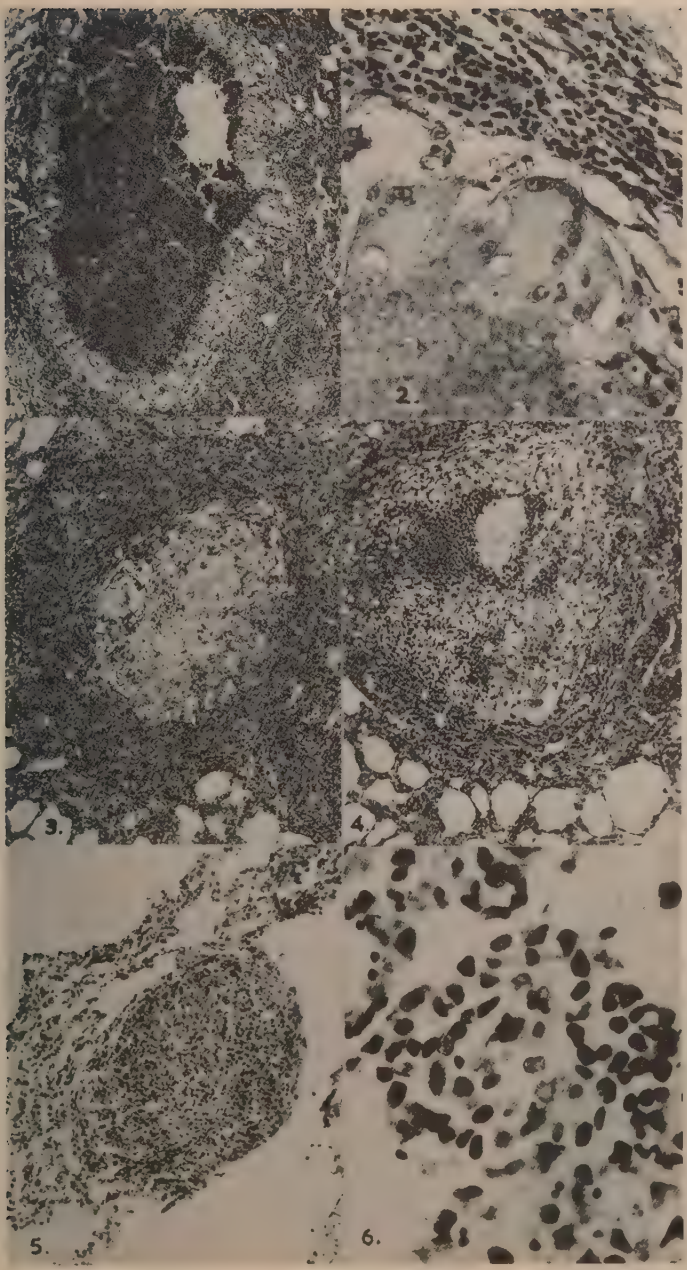

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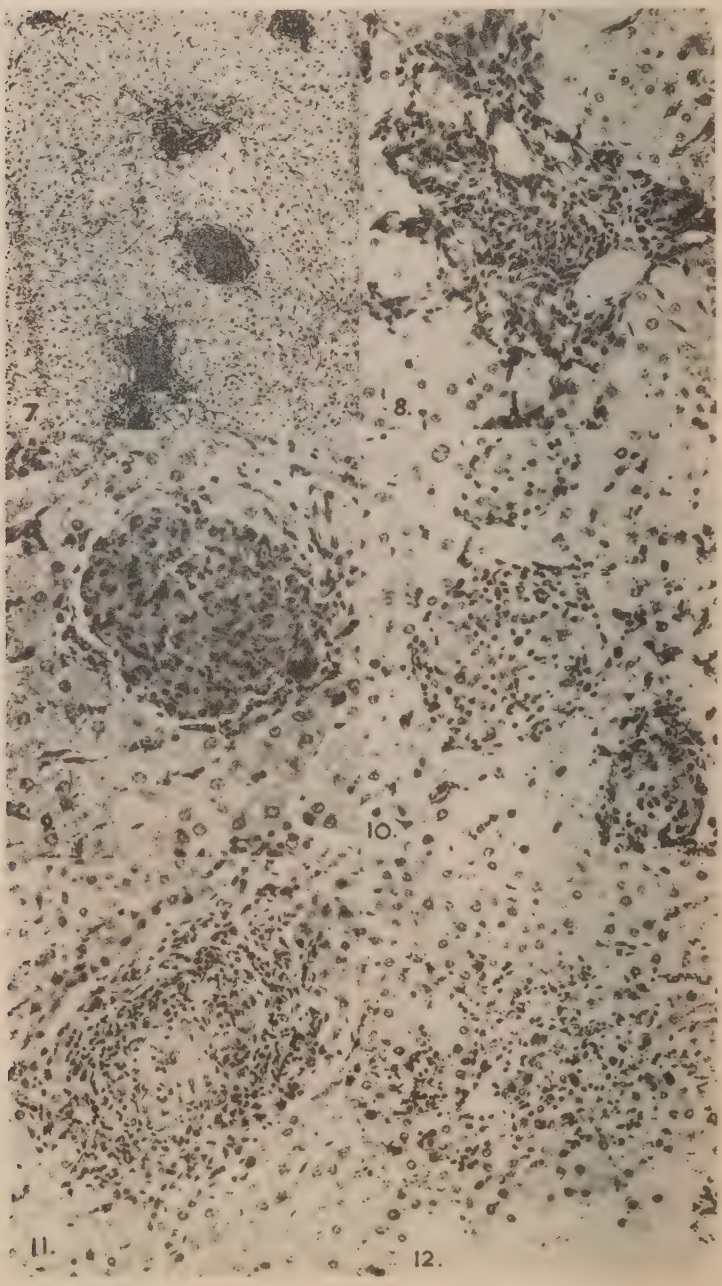
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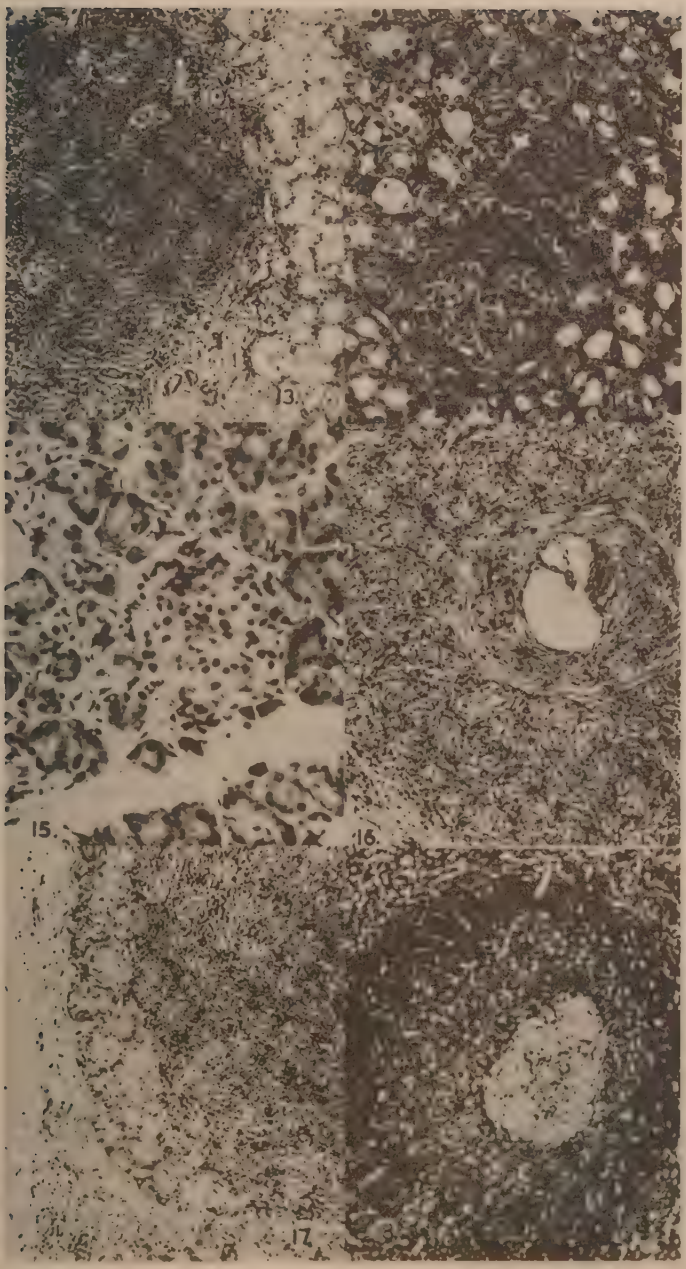
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INCIDENCE OF CARPAL HYGROMAS IN DAIRY CATTLE INFECTED WITH *BR. ABORTUS* AND MAINTAINED IN AN ISOLATION COMPOUND

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INTRODUCTION

Although the possibility of an association between *Br. abortus* infection and hygroma of the carpus has long been recognised, no evidence has been presented to show conclusively that *Br. abortus* is an etiological factor.

In Sweden, Bergman and Agren (1923) reported that hygromas were often found in herds in which infectious abortion was present. Boyd, Delez and Fitch (1930) frequently noticed that cows affected with hygromas were often positive reactors to the agglutination test for *Br. abortus*, and they isolated the organism from hygromas in two cows. These authors were of the opinion that the data they had accumulated indicated that *Br. abortus* was an associated etiological factor in the development of carpal hygromas.

The possibility of such a relationship has been considered in Great Britain. McDiarmid (1954) has isolated the organism from carpal hygromas. Cattle admitted to the Surgery Department at the Royal Veterinary College for the treatment of excessively large hygromas have been found frequently to react to the *Br. abortus* agglutination test.

MATERIALS AND METHODS

Animals under observation. The cattle assembled at Compton for an experiment to determine the degree and duration of immunity in cattle vaccinated with S.19 presented an opportunity for observations to be made on the incidence of carpal hygromas in the animals infected experimentally with *Br. abortus*. The cattle were mainly Ayrshires, and, apart from a short period at grass after weaning, they were maintained in isolation units, 12 animals per unit, in a large compound. During the day the animals were allowed out to exercise in a covered yard with a concrete floor. Overnight they were bedded down in their stalls.

Time of observations. Not until the cattle had been confined within the isolation units for approximately three years, did it become apparent that the incidence of carpal hygromas was increasing. The first series of observations was made in February 1954, when the cattle were about 5 years of age, and the second series in the corresponding month of 1955. At

TABLE 1

INCIDENCE OF CARPAL HYGROMAS IN CATTLE MAINTAINED IN AN ISOLATION COMPOUND

History of vaccination with Br. abortus *Strain 19*	*Infected with* Br. abortus						*Non-infected*					
	Number of cattle		*No. of limbs showing evidence of hygroma*		*% of limbs showing evidence of hygroma*		*Number of cattle*		*No. of limbs showing evidence of hygroma*		*% of limbs showing evidence of hygroma*	
	1954	*1955*	*1954*	*1955*	*1954*	*1955*	*1954*	*1955*	*1954*	*1955*	*1954*	*1955*
Vaccinated at 6 months	20	20	6	3	15	7·5	48	27	5	5	5·2	9·3
Vaccinated at 18 months	20	19	4	5	10	13	50	26	5	5	5	9·6
Vaccinated at 6 and 12 months ..	20	19	1	4	2·5	10·5	50	28	5	10	5	18
Vaccinated at 6, 12 and 18 months	20	20	2	9	5	22·5	56	34	8	6	7·1	8·8
Non-vaccinated controls	10	10	3	4	15	20	30	19	3	3	5	7·9

the time of the observations some of the cattle in each of the groups (see Table 1) had been experimentally infected five months previously by conjunctival instillation of either 15 million or 150 million *Br. abortus*.

Examination of the fore limbs for hygromas. At first an attempt was made to classify the enlargements according to the nature of the swelling, but it was found that such a procedure was unduly complicated and not really essential for the purpose of this investigation. Consequently all animals showing clinical evidence of an enlargement over the anterior aspect of the carpus were considered as being affected with hygroma.

RESULTS

To study the effect of *Br. abortus* infection on the incidence of hygromas, the percentage was calculated of fore limbs which showed evidence of hygromas in the five experimental groups which were infected with *Br. abortus*, and also in those groups which were not infected. From the figures obtained, (Table 1) the mean percentage of the incidence was calculated in the infected and non-infected animals. It was then observed that the mean percentage of limbs of the infected cattle showing hygromas in 1954 and 1955 was 9·5 and 14·7 respectively. Although there is an apparent higher incidence on both occasions, in the animals infected with *Br. abortus*, statistical analysis of the results (Table 1) from which these figures were calculated shows that this increase is not significant ($p>0.05$).

If the incidence of the condition in the infected vaccinated cattle is compared with that of the infected non-vaccinated animals, it is seen that although the incidence in the latter group is greater by 7 per cent in 1954 and 6·6 per cent in 1955, statistical analysis of the results from which these figures were calculated shows that there is no significant difference between the incidence of the hygromas in the two groups ($p>0.07$), thus showing that vaccination with strain 19 does not appear to affect the incidence of hygromas.

The total incidence of hygromas in vaccinated animals which were resistant to *Br. abortus* infection and that in similar cattle which did not resist infection in 1954 and 1955 is shown in Table 2. From this table it is seen that there is no appreciable difference in the incidence of hygromas in the resistant animals when compared with that in non-resistant animals.

DISCUSSION

Carpal hygromas occur mostly in housed cattle particularly those confined to places with concrete floors or other hard surfaces (Frank, 1947). They arise as a result of repeated bruising of the anterior aspect of the carpus but may follow a single severe trauma. Although there are suggestions in the literature that *Br. abortus* is an etiological factor associated with carpal hygromas in cattle the findings recorded in this paper do not support that view. This conclusion is reached since it has been observed that there is no

TABLE 2

INCIDENCE OF CARPAL HYGROMAS

Cattle resistant and those not resistant to an experimental infection with *Br. abortus**

Infecting dose of Br. abortus	*No. of limbs affected with hygromas in resistant cattle*	*No. of limbs affected with hygromas in non-resistant cattle*
15 million	11/13	4/7
150 million	6/6	7/8

*Resistant animals were those from which no *Br. abortus* were isolated at parturition.

significant difference in the incidence in animals infected with *Br. abortus* compared with non-infected animals. The conclusion is also substantiated by the fact that the incidence in cattle which were resistant to *Br. abortus* infection was similar to that in animals which became infected by an experimental challenge dose of *Br. abortus*. Furthermore, if *Br. abortus* were an etiological factor associated with the condition, it might be expected that the incidence would have been significantly less in cattle vaccinated with avirulent S.19 vaccine, but this was not so.

CONCLUSIONS

The incidence of carpal hygromas in cattle infected experimentally with *Br. abortus* was not significantly higher than in control uninfected cattle maintained with them in an isolation compound. The incidence of hygromas was as high in cattle vaccinated one or more times with S.19 as in unvaccinated cattle.

Hygromas were as common in cattle which were resistant to *Br. abortus* as measured by failure to recover the organism at parturition following experimental inoculation, as in non-resistant animals.

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QUANTITATIVE STUDIES ON THE CONTAMINATION OF PASTURE HERBAGE WITH HUSK WORM LARVAE

By

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INTRODUCTION

Pasture herbage is the essential vehicle in the transmission of lungworm infection. It has been shown in field experiments (Michel and Parfitt, 1955, 1956) that the concentration of larvae on the herbage determines the size of the infection established in a susceptible calf grazing on it. At the same time there is no close connection between the number of larvae being passed on to a pasture and the resulting herbage infestation. It follows that the process whereby larvae in the faeces become infective larvae on the herbage, the process of translation as it has come to be called, is of over-riding importance in the transmission of infection. This is confirmed by the results of Spedding and Michel (1956) which also suggest that mechanical spreading of faeces is of importance in the process of translation.

In the course of observations on the bionomics of the larvae of *D. viviparus* (Michel and Rose, 1954) it was found that only a negligible proportion of the larvae present in a faecal pat reached the herbage. The very small number that did were so close to the faeces that it was considered that they would not have been picked up by the grazing bovine which is known to avoid faeces. It was suggested that it was the dissemination of faeces and not the migration of the larvae which made these available to the grazing animal and in experiments on grass grown in boxes appreciable numbers of larvae were only recoverable from the herbage of those boxes over which the faeces had been thinly spread with a spatula. In the field the spreading of faeces can be brought about by numerous agencies.

The consistency of the faeces largely determines the extent to which they are disseminated, the feet of grazing animals may assist in spreading faeces and birds are often seen to break up and scatter faecal pats. The operations of husbandry, whether with the harrows, the mower or with other implements which are applied to render the pasture more uniform are all effective in spreading faeces and are indeed largely directed to this end.

The experiments to be described were carried out to enquire more closely into the effects on translation of such factors. It was also desired to determine whether infective material on a pasture which was inaccessible to the grazing animal could, after a lapse of time, still be made available by the intervention of factors tending to disseminate the faeces.

METHODS

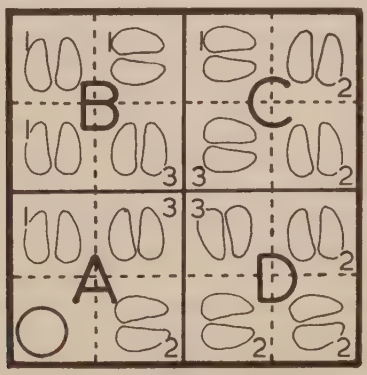
The effects of five different factors were observed on the spreading of faeces and larvae over the herbage. These were the consistency of the faeces, mechanical agencies, climatic conditions, age of faeces and larvae and the possible role played by birds. Five repetitions of the experiments were carried out starting on April 26th, July 19th, September 6th, October 11th and January 8th respectively. Faeces were collected from a number of infected calves and thoroughly mixed to ensure a uniform distribution of larvae. A constant amount of 1¾ lb. was used to contaminate each experimental plot of grassland. These plots were 1 yard square and were laid out on a cocksfoot and S100 ley in its second year. The grass was cut to a height of 3 to 4 inches before putting on the faeces.

Consistency of faeces. The effect of three different consistencies of faeces designated "solid," "semi-fluid," and "fluid" was compared. "Solid" faeces were as passed by stalled calves and of between 20 and 25 per cent dry matter. "Semi-fluid" and "fluid" were prepared from "solid" by the addition of 350 and 800 ml. of water respectively to each 1¾ lb. portion. In contaminating the plots the aim was to cover as great an area as the consistency of the faeces permitted. The faeces were poured or dropped from a bowl from approximately 3 ft. above the ground. Four plots were used for faeces of each consistency.

Mechanical agencies and age of faeces. The faeces were such that when dropped they formed a pat but were sufficiently moist to be readily spread (1¾ lb.: 250 ml. water). For purposes of sampling the plots were divided into 4 squares designated A, B, C and D. A pat of the faeces was placed in the outer corner of square A in each of 32 plots. On 8 plots the faeces were spread immediately; faeces on a further 8 plots were

Fig. 1.

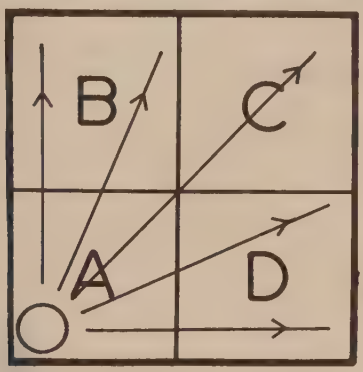
Spreading of faeces with the cow's foot



The small squares numbered 1, 2, and 3, indicate the three pathways followed in spreading the faeces over the herbage. The cow's foot was pressed once into the faecal pat in the corner of square A, then once in each of the small squares along pathway 1. This was repeated for pathways 2 and 3. Thus the first imprint along each route was in square A.

spread at weekly intervals. Two methods of spreading were employed. On 4 plots each week, the faeces were spread by means of a cow's foot. The plot was divided into nine squares measuring nine inches by nine inches. The foot was pressed into the faecal pat and then once in each small square along pathway 1 (Fig. 1). It was then pressed in the pat twice more, once before traversing pathway 2 and again before traversing pathway 3 in the same way. On the other 4 plots the faeces were spread each week with a small handfork. The pat was divided into five almost equal parts and the faeces distributed along each one of the pathways (Fig. 2) by four strokes of the fork.

Fig. 2.
Spreading of faeces with the handfork



A pat of faeces was dropped in square A. and then spread over the herbage of the plot by means of a small handfork, along the five pathways indicated in the diagram.

Distribution by birds. Pats of faeces of the same consistency as those used in the above experiments were put on to the middle of eight more plots; four were covered by means of wire netting to protect them from birds ("protected") and the other four were left uncovered ("unprotected").

Sampling of herbage and faeces. At weekly intervals all the herbage was cut at approximately half an inch from the soil surface from one plot of each experimental treatment. Great care was taken to ensure that no pieces of faeces were picked up. In the experiments on consistency of the faeces the whole of the herbage on a plot was examined as a single unit. Where the faeces had been spread by mechanical means, each plot had been divided into four quarters, A, B, C, and D and the herbage from each was examined separately. The plots with the "protected" and "unprotected" faeces were divided into two areas, one immediately around the pat two inches in diameter termed the "inner" area and the other which constituted the remainder of the plot, the "outer" area. The herbage from the two areas was examined separately. Samples of faeces were taken for as long as possible. It was possible to obtain 10 g. from the pats every week, but where the faeces had been spread, samples

could not always be obtained for the whole period of the experiment.

The enumeration of larvae in faeces and on herbage was effected by the methods described in an earlier communication (Michel and Rose, 1954).

RESULTS

The total numbers of larvae recovered from the herbage of the experimental plots over the period of 4 weeks are represented in Fig. 3. (Exp. 1 to 5). The histograms designated "Pat A" represent the larvae from plots with the "protected" pats, and those designated "Pat B" the larvae from the plots with the "unprotected" pats.

Fig. 3.
Translation of larvae from faeces to herbage

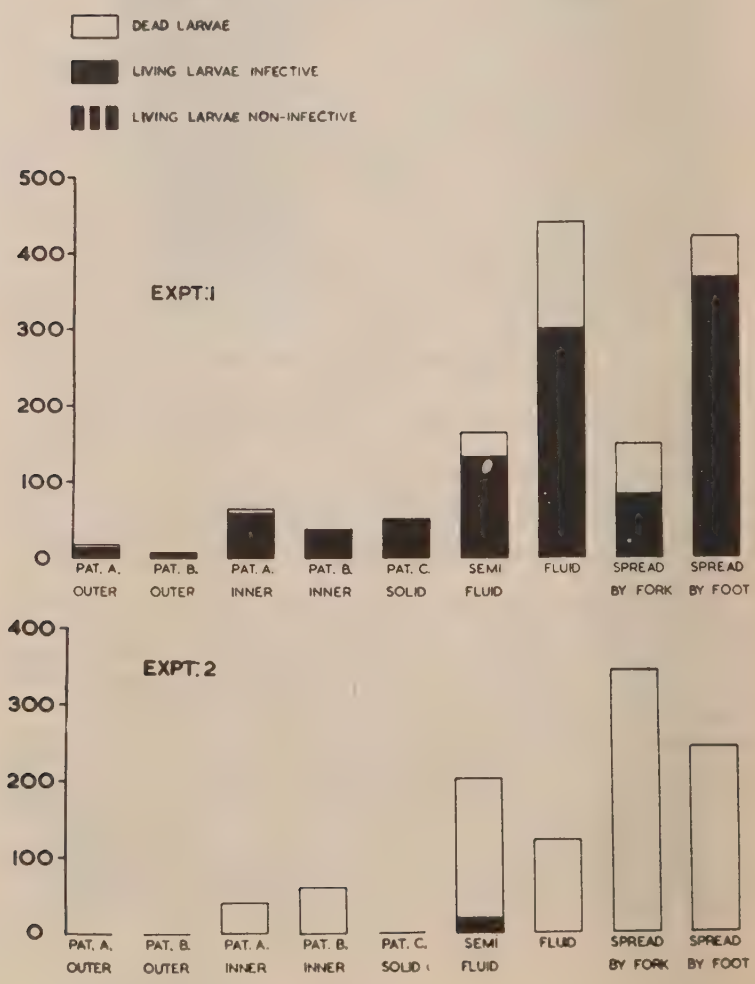


Fig. 3 (*continued*)
Translation of larvae from faeces to herbage

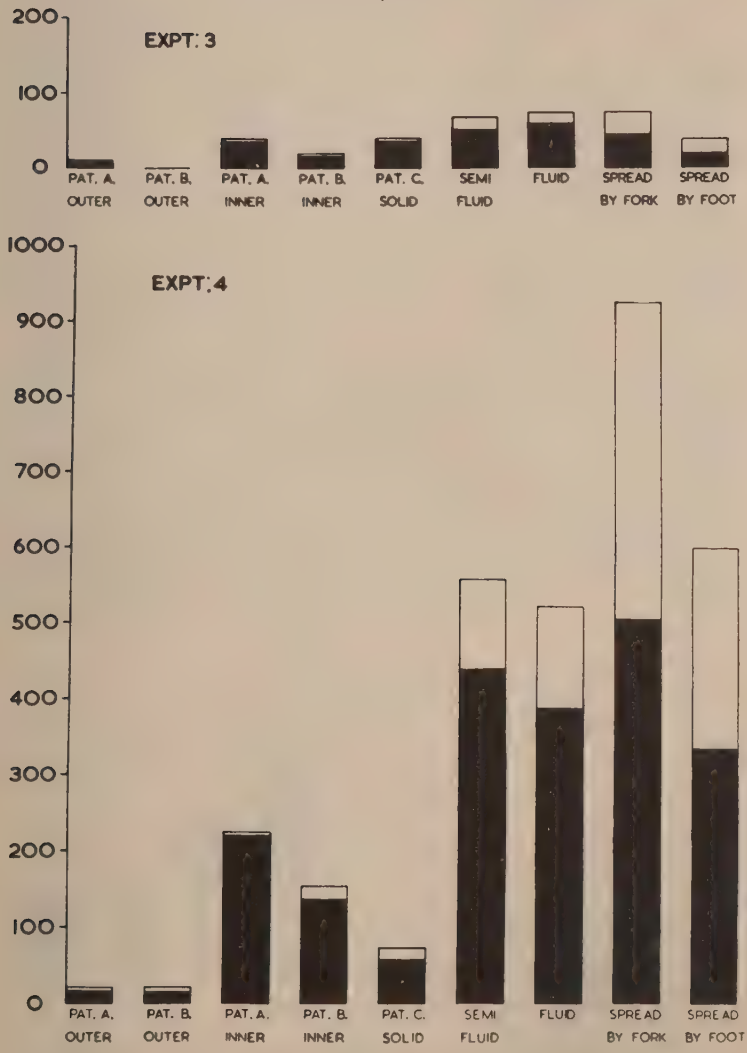
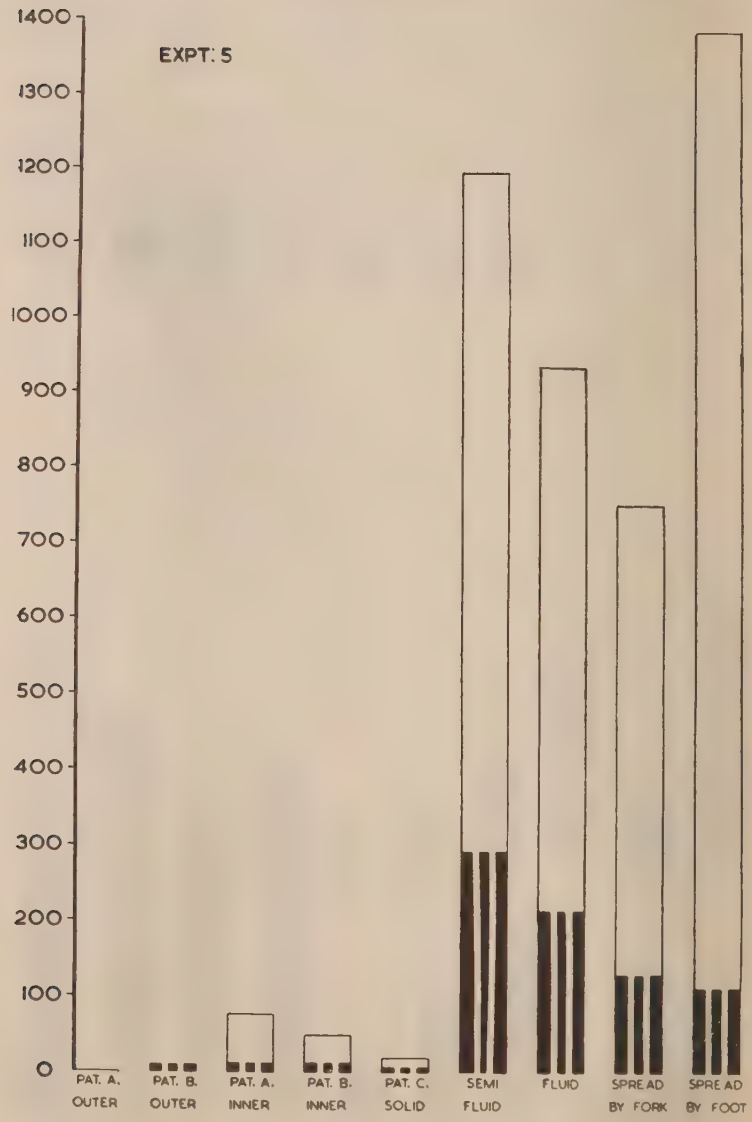


Fig. 3 (*continued*)



The total number of larvae recovered from the herbage of the experimental plots over a period of four weeks, showing the effect of consistency of faeces, mechanical agencies and birds on the dissemination of larvae over the herbage in five different climatic environments.

It can be seen that far more larvae can be recovered when faeces are spread over the herbage than when they are deposited as a pat.

The number of larvae reaching the herbage is influenced to an appreciable extent by the consistency of the faeces as can be seen on comparing the histograms "solid," "fluid," and "semi-fluid" (Fig. 3). Far more larvae were recovered from the plots on to which the fluid and semi-fluid faeces were dropped than from the plots with the solid faeces. This was mainly as a result of the ease with which the fluid and semi-fluid faeces could be spread to contaminate most of the herbage of the plot, whereas the solid faeces could only be dropped as a pat contaminating a much smaller area. Of the few larvae which did pass from the pats the majority remained in close proximity to the faeces and were recovered from the herbage of the inner area. It would seem, therefore, that when animals excrete diarrhoeic faeces which are usually spread over a wide area, far more larvae will reach the herbage than when the animals excrete drier faeces which are deposited as a pat, unless the pat is spread by some mechanical agency.

That mechanical spreading of the faecal pats is an effective means of distributing larvae over the herbage is apparent from the histograms "Spread by Foot" and "Spread by Fork." (Fig. 3). More larvae were recovered from the plots over which the faeces had been spread either by means of a small fork or using a cow's foot than from those on which the faeces were in the form of a pat. Larvae were recovered from each of the small areas A, B, C, and D into which the plots had been divided, although in general more were recovered from square A than from any of the other squares. In some instances the total recovered from B, C, and D, was less than the number from square A alone (Table 1).

TABLE 1

THE DISTRIBUTION OF LARVAE OVER THE HERBAGE

When faeces were spread by means of a handfork or a cow's foot

Experiment No.	*Living larvae recovered from herbage*			
	Spread by fork		*Spread by foot*	
	A	*B+C+D*	*A*	*B+C+D*
1	193	239	383	133
2	0	0	0	0
3	89	135	18	30
4	588	577	499	442
5	21	108	27	90

It will be seen from Fig. 3 that there were marked differences in the numbers of larvae recovered from the herbage in the five repetitions which were started in April, July, September, October and January respectively. Meteorological data for these periods is presented in Table 2. It does not seem unreasonable to suppose,

TABLE 2

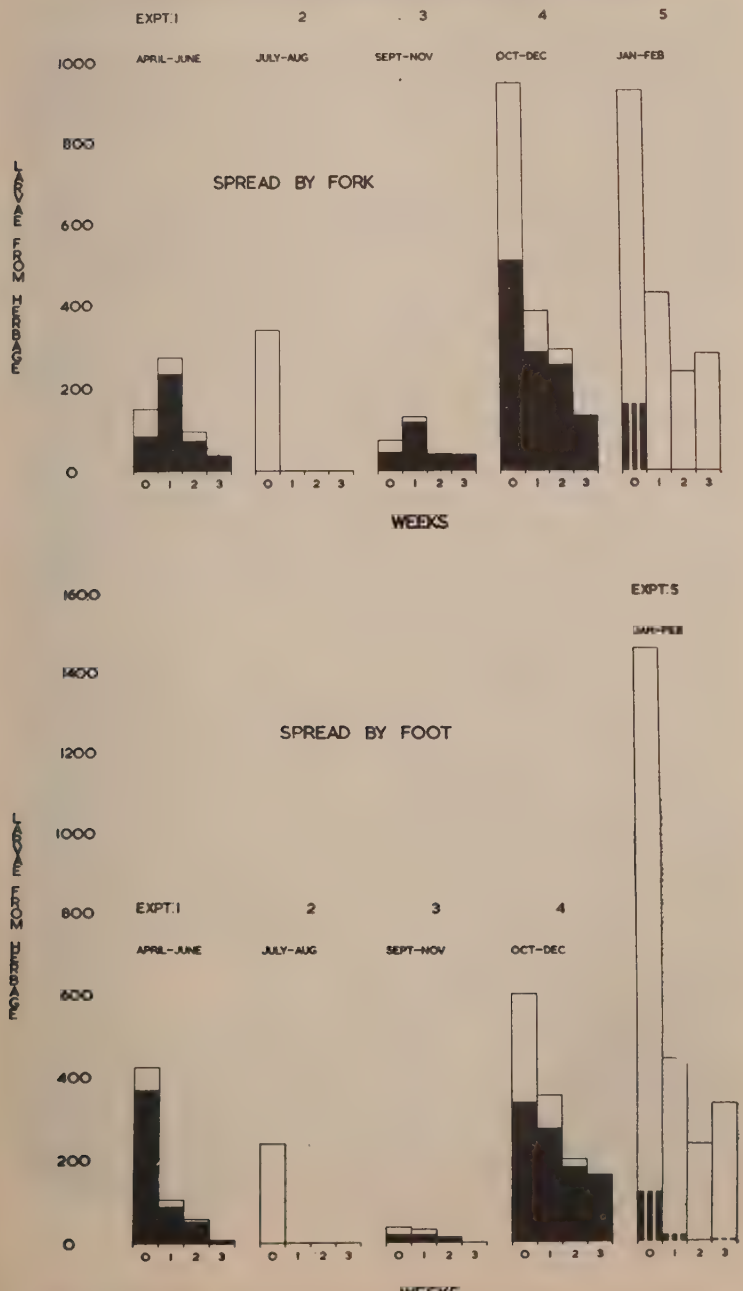
METEOROLOGICAL DATA FOR THE PERIOD APRIL 1955 TO FEBRUARY 1956

Month	*Temperature °F.*		*Total monthly rainfall Inches*	*No. of days when temperature was 32°F. or less*	
	Mean monthly maximum	*Mean monthly minimum*		*Air*	*Ground*
1955 April	61·2	44·1	0·4	1	7
May	58·9	41·9	2·4	—	—
June	69·9	51·5	1·85	—	—
July	74·1	54·5	0·25	—	—
August	79·6	53·5	1·49	—	—
September ..	66·1	47·1	1·84	—	—
October	54·7	38·6	3·23	4	10
November ..	48·9	36·8	1·54	9	15
December ..	49·5	38	2·07	10	17
1956 January	42·1	31·6	3·96	17	23
February ..	37·9	27·8	0·15	23	26

therefore, that climatic factors in addition to mechanical spreading play a part in distributing larvae over the herbage. It was observed that the state of the spread faeces and the rates at which they disintegrated varied. In the winter (Exp. 5) the spread faeces and the herbage were wet almost continually except for brief periods when the faeces were frozen. By the end of 4 weeks no spread faeces were visible on the herbage and the large number of larvae recovered was probably associated with this disintegration of faeces. In the spring and late autumn the spread faeces were moist for most of the time and gradually disintegrated, although not as rapidly as in the winter, and fewer larvae were recovered from the herbage (Fig. 3 Exp. 1 and 4). The smallest numbers of larvae were recovered from the herbage during the summer and late autumn (Fig. 3, Exp. 2 and 3) when the faeces were dry within a few hours of being spread and underwent no visible disintegration even though in autumn they were subsequently wetted. Examination of

Fig. 4

The effect of age of faecal pats and larvae on spreading



Numbers of larvae recovered from the herbage of plots over which were spread faecal pats which were 0, 1, 2, and 3 weeks old at the time.

some of the small pieces of faeces revealed the presence of many larvae; in the summer they were all dead but in the early autumn many were living.

The numbers of larvae recovered from the herbage of plots on which the pats were 1, 2, and 3 weeks old at the time of spreading are represented in the histograms (Fig. 4). It should be noted, however, that all the larvae recovered during the winter were non-infective.

It can be seen that larvae did reach the herbage when these pats were spread. Again the numbers of larvae recovered varied at different times of the year. These differences were largely due to the extent to which the faeces could be spread and partly to the different rates of disintegration of the spread faeces.

In the winter and later autumn, pats which were 1, 2 and 3 weeks old were very wet and were easily spread. At these times considerable numbers of larvae were recovered from the herbage. (Fig. 4, Exp. 4 and 5) even from plots on which the pats were 3 weeks old when spread. In the spring and autumn the pats dried out and although the centre of the pat remained moist a thick dry layer formed over the surface which made spreading more difficult. The pat could only be broken into small pieces which were scattered over the plots. Some larvae did pass on to the herbage (Fig. 4, Exp. 1 and 3), but not as many were recovered as in the winter and late autumn. In the summer the pats dried out so thoroughly within a few days that it was impossible to spread them. Living larvae were, however, present within the pats.

The faecal pat may thus act as a reservoir of infection which can still be made available to the grazing animal after a lapse of weeks by the intervention of mechanical factors providing that climatic conditions have left it in a condition such that it can be spread.

The effect of birds on the dissemination of larvae did not appear to be of any practical significance. Only on two occasions during the autumn were there indications that the pats of faeces had been attacked by birds, although in a nearby field during the summer many pats were seen to have been broken up and scattered by birds. Examination of these pats showed them to be full of insect life for which source of food the birds had doubtless been searching. These pats were, however, dry and old and it is unlikely that many larvae would have been surviving in them.

There was no appreciable difference in the numbers of larvae recovered from samples of faeces from the "protected" and "unprotected" pats, nor was there any significant difference in the numbers of larvae recovered from the herbage. (Fig. 1, pats A and B). The few which were recovered were nearly all from the herbage close to the faeces which would suggest that they had either migrated or been washed out of the pats. It would seem that birds may occasionally spread faeces and larvae, but no evidence was obtained to indicate that they are at all important in this respect.

DISCUSSION

It is clear that the larvae have a negligible chance of reaching the herbage unless the faeces themselves are spread in some way. To think in terms of a clear-cut distinction between larvae in faeces and larvae on the herbage is, therefore, erroneous. Larvae get on to the herbage when faeces do so. The larvae are in the faeces and the faeces are on the herbage. The concept of larvae on the herbage as opposed to larvae in the faeces implies that the faeces are so thinly smeared or so thoroughly disintegrated by the rain that they are no longer recognisable or separable from the herbage. Such a distinction between larvae in recognisable particles of faeces and larvae in films of faeces that are not recognisable is of itself meaningless, the only proper difference being between larvae available to the grazing animal and larvae not available. The validity of experimental results would seem to depend on the extent to which these alternative distinctions are synonymous.

In a comparison of the concentration of larvae on herbage grazed by bovines and in samples cut with scissors (Michel, 1955), it was shown that the bovine recovered fewer larvae than the observer even when considerable pains were taken to avoid faeces. The differences, however, were not large and implied a quantitative rather than a qualitative difference between selective grazing and random sampling; experiments on the grazing behaviour of cattle in relation to faeces (Michel, Ollerenshaw and Rose, 1956) showed that the cattle will graze contaminated herbage unless the faeces are conspicuous.

While it is clear that the process of translation is dependant on the spreading of faeces over the herbage, the development of the larvae to the infective stage and their initial survival is of equal importance. However, the periods when conditions are most conducive to the transference of larvae onto the herbage are not necessarily conducive to development and survival. The maximum numbers of larvae were recovered from the herbage during the winter, but these larvae were either non-infective or dead. The first and second stage larvae are, as shown by Rose (1956), more susceptible to freezing than are the infective larvae. In winter, therefore, when the rate of development is retarded, the larvae remain for much longer at a stage susceptible to low temperature so that all on the herbage succumb though a few in faecal pats persist and eventually develop to the infective stage.

In the spring and autumn all the living larvae recovered were infective. The infective larvae, however, are no more resistant to desiccation than the non-infective and, in consequence, all the larvae recovered from the herbage in summer were dead. It should perhaps be mentioned in this connection that the summer of 1955 was unusually hot and dry; none the less some larvae in the pats survived for three weeks.

Larvae in a faecal pat, though not available to the grazing animal, are less exposed to adverse climatic factors than larvae on the herbage. Where the structure of the pat permits of its spread by mechanical agencies such sheltered larvae may be made available at a later stage. The faecal pat may thus be regarded for a limited period as a potential reservoir of infection. The proportion of total available larvae may be increased by the intervention of those operations of husbandry which aim at the dissemination of faecal pats.

CONCLUSIONS

Experiments were carried out on small plots of grassland to study the effect of factors tending to disseminate the faeces on the transference of larvae from faeces to herbage.

The extent to which the herbage could be contaminated varied with the consistency of the faeces.

Mechanical agencies were used to spread faeces and larvae.

The numbers of larvae recovered from herbage over which faeces had been spread were associated with the effect of climatic factors on the disintegration of the spread faeces.

Where pats 1 to 3 weeks old were in such a condition that they could be spread by mechanical means, larvae were recoverable from the herbage.

The implications of the conclusion that larvae only reach the herbage when faeces do so are discussed.

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THE VIRUS OF VESICULAR STOMATITIS IN SMALL EXPERIMENTAL HOSTS

I. White Mice, Cotton Rats, Chick Embryos and Young Chickens

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INTRODUCTION

One of the main purposes of these studies with the virus of vesicular stomatitis was to pave the way for fresh investigations into the possibility of using mice and chick embryos as hosts for the propagation of the virus of foot-and-mouth disease. These two viruses produce a clinical picture which is very similar in naturally infected hosts such as cattle and swine and in experimentally infected guinea-pigs. In the case of the virus of vesicular stomatitis, mice and chick embryos are also susceptible to infection and are commonly used as experimental hosts, but when these studies commenced in 1947 the guinea-pig was recognised as the only small experimental animal available for use in large numbers in work with the virus of foot-and-mouth disease. The fact that methods of regularly infecting white mice, chick embryos and chickens with the virus of foot-and-mouth disease have now been developed (Skinner, 1951, 1954) was, as will be pointed out later, in no small way due to the experience gained of the behaviour of the virus of vesicular stomatitis in these hosts.

The observations in this and the following paper (Skinner, 1957) were completed in 1951. The delay in placing them on record has been due to preoccupation in the interim with the studies on the virus of foot-and-mouth disease to which they gave rise.

The available strains of vesicular stomatitis virus were of comparatively recent field origin and preliminary experiments with them led to a wider range of investigations which showed that the properties of these strains resembled closely those described for the few strains of much earlier origin which most other workers had used in their experimental studies. There were however several aspects of the work which disclosed properties hitherto unrecorded and in the main it is these which have been selected for presentation. A very important finding was the readiness with which daylight inactivated the virus during normal laboratory procedures. An account of the investigations into this phenomenon has already been published (Skinner and Bradish, 1954).

Reference is made to the majority of the papers published on the subject of these studies since 1932. An adequate review of the literature published prior to this was made by Wagener (1933).

MATERIALS AND METHODS

Strains of virus. Two strains originating from the U.S.A. (Ind.C and NJ.M), and one from Mexico (MEX.1) have been the sole sources of virus. The Ind.C strain (Indiana type) was isolated from a naturally infected horse in Colorado in 1942. Heinz (1945) has given an account of the epidemic occuring at that time. The NJ.M strain (New Jersey type) was recovered from swine in the first outbreak under natural conditions to be recorded in that species. This occurred in Missouri in 1943 (Anon, 1943; Sanders and Quin, 1944). Both these strains have been used by American workers in experimental studies (Shahan, Frank and Mott, 1946).

When received from the U.S. Department of Agriculture these strains had been passaged at varying intervals in different hosts and their history in this respect was stated to be as follows.

Strain	*Field sample*	*Experimental serial passages of field sample*
Ind.C	Horse	Cattle 1 (i.e. one passage) — Guinea-pig 5 — Cattle 1 — Guinea-pig 1 — Cattle 1
NJ.M	Swine	Guinea-pig > 10 — Swine 1 — Cattle 1 — Guinea-pig 1 — Swine 1 — Guinea-pig 9 — Cattle 1

Tongue epithelium from the final cattle passage of each strain was received at this Institute in 1944. Before the strains were used in the present studies five more serial passages of each had been made in cattle.

The MEX.1 strain was recovered from a field sample from a calf in Mexico in 1947. It was studied by Brooksby (1949) and classified as Indiana type.

Infectivity titration procedure. To titrate the infectivity of a tissue a 1 in 10 w/v ground suspension was prepared in 0.15 M NaCl buffered at pH 7.6 by the addition of 0.005M phosphate salts. This suspension was then clarified by centrifugation and the supernatant was taken as the initial virus suspension (10^{0} dilution) from which ten-fold dilutions in the same suspending medium were prepared for titration in guinea-pigs, mice or chick embryos. To prepare a Seitz EK filtrate a 1 in 25 w/v ground suspension in equal parts of the buffered saline and Hartley's broth was first prepared and the filtrate was taken as the initial virus suspension. Throughout these procedures precautions were taken against unnecessary exposure of virus suspensions to daylight.

Infectivity titres are expressed as the 50 per cent end-point dilution of the initial virus suspension calculated by the method of Reed and Muench (1938).

RESULTS

WHITE MICE

The susceptibility of white mice to clinical infection following intracerebral inoculation of the virus of vesicular stomatitis was first demonstrated by Cox and Olitsky (1933a and b) and by Olitsky, Cox and Syverton (1934) who used virus strains of Indiana and New Jersey type which had been passaged intradermally in guinea-pigs more than 700 times. In this and in further work (Olitsky, Sabin and Cox, 1936) the different routes of inoculation of the mouse were compared and comparisons were also made between the susceptibility of mice aged two weeks and one year. Titrations of suspensions of mouse brain tissue based on intracerebral

inoculations showed that the minimal infective dilution was the same for mice of both these ages but when the intranasal route was used the virus dose required to infect the older mice was at least 100 to 1000 times greater than that required to infect the younger mice. Only the younger mice could be infected regularly by the intraperitoneal, subcutaneous and intramuscular routes. Infection occurred after feeding infected food and the importance of avoiding accidental infection of experimental mice by the intranasal route was stressed. Sabin and Olitsky (1937a and b, 1938) made later a valuable detailed study of the fate of the virus in mice infected by the intracerebral, intranasal, intramuscular, intraneural, intra-ocular and subcutaneous routes. The strains of virus employed were shown to be essentially neurotropic in the mouse. The only tissue other than that of the central and peripheral nervous systems in which the virus appeared to multiply was the skeletal muscle of young mice at the site of intramuscular inoculation. Virus was not demonstrated in the blood of infected young or old mice and was rarely found in the viscera. Slavin, Hale and Berry (1946) confirmed some of these findings and also showed that a passive immunity was transmitted by an actively immunised female mouse to her offspring by means of her milk.

In the present studies with the Ind.C and NJ.M strains of the virus of vesicular stomatitis, white mice could be infected readily by the intracerebral route with development of clinical disease. Previous passage by other routes of inoculation in cattle, guinea-pigs, chick embryos or young chickens did not affect the issue. However, for experiments in mice involving the accurate quantitative estimation of virus it was considered preferable to employ strains which had been passaged serially in mice by intracerebral inoculation.

One of the problems which appeared to require further investigation was the readiness with which infection spread amongst mice in contact with each other. Further information was also required in respect of the readiness with which mice could be infected intranasally.

Experimental Procedure

The white mice were from a colony maintained at this Institute, and originated from the Parkes strain obtained from the National Institute for Medical Research, Mill Hill, London. The exact age of all mice used was known. In routine titrations the mice were aged 3 to 6 weeks and within a single experiment ages did not usually vary more than a week. In the course of the earliest infectivity titrations the comparative susceptibility of different litters to infection was investigated by inoculating the mice within each of several litters with the full range of virus dilutions. When it was found that there was no variation in the susceptibility of different litters this procedure was changed for one in which the mice in any experiment were selected from litters of similar size in the age group required. All mice were first pooled in a single container from which they were taken at random for distribution throughout the various groups of the experiment. Each group was kept in a separate tin in order to minimise any variation in the periods of incubation of the disease in mice in contact with each other which, as will be shown later, occurs with

variation of the infective dose. Serial passaging of virus strains in mice was made either by the intracerebral or the intranasal route. Inoculations were made under light anaesthesia induced by ether. In the intracerebral method 0.03 ml. was inoculated into the right cerebral hemisphere. The dose instilled intranasally was about 0.015 to 0.03 ml. according to the size of the mouse. Mice were examined at least once daily after inoculation; usually twice daily over the period during which clinical signs were expected to develop.

In mice younger than 12 weeks the onset of paralytic symptoms was usually followed within a few hours by death. Sudden noise often precipitated a fatal spasm during which there was extreme extension of the hind limbs and flexion of the head. Dead mice were removed and a note made of any evidence of cannibalism. Mice showing obvious clinical signs were killed and the brains from such mice, removed as soon as possible by a sterile technique, were the sole source of infective mouse tissue. Daily observations were continued for about three weeks. Surviving mice were usually tested for immunity by inoculation of the left cerebral hemisphere with 100 or more cerebral ID50 of the homologous mouse passaged strain. This test detected mice which had suffered a subclinical infection, but the calculation of the 50 per cent end-point dilutions for estimation of infectivity titres was based only on clinical infections.

The Effect of Age on Infection by the Intracerebral and Intranasal Routes

Intracerebral route. A correlation was observed between the number of ID50 doses inoculated intracerebrally and the interval before the appearance of clinical signs. The data obtained on this point from intracerebral titrations of the Ind. C mouse strain in mice aged 3 to 6 weeks is presented in Table 1. At this age the majority of mice had developed clinical signs by the third day. The incubation period was sometimes as short as two days in those receiving only one ID50. An incubation period as short as this in mice aged 1 to 2 years was observed only when the dose of virus was more than 100 ID50. Similar observations were made with the NJ.M mouse strain.

As the data in Table 1 indicates, when a dilution factor of 10 was used in infectivity titrations the 50 per cent end-point was covered

TABLE 1

THE INCUBATION PERIOD IN MICE AGED 3–6 WEEKS

After intracerebral inoculation of virus of the Ind.C intracerebral mouse strain

Log. of ID50 inoculated	*Approximate percentage of mice which had developed clinical signs*			
	1st day	*2nd day*	*3rd day*	*4th—7th day*
—1·0	0	0	0	0
—0·5	0	0	5	10
0·0	0	10	40	50
0·5	0	40	90	90
1·0	0	70	100	100
1·5	0	90	100	100
2·0	0	100	100	100

frequently by dilutions giving an infection rate of 0/5 and 5/5. This occurred with mice of all ages and with strains other than those passaged in mice. A dilution factor of 3.2 (Log. 0.5) was, therefore, sometimes used in intracerebral infectivity titrations to increase accuracy.

In two series of intracerebral titrations of 1 in 10 suspensions of brain tissue of mice infected with the NJ.M intranasal strain, there was evidence that mice 1 to 2 years old could not be regularly infected intracerebrally by a virus dose containing less than 10 i/c ID_{50} for younger mice. In the first series the respective 50 per cent end-point dilutions in mice aged 3, 5, 7 and 20 weeks, and over one year, were $10^{-5.4}$, $10^{-4.8}$, $10^{-5.4}$, $10^{-4.5}$ and $10^{-4.2}$, and in the second series in mice aged 6 to 7 weeks and over one year, $10^{-5.5}$, and $10^{-4.0}$. Similarly in a titration of a suspension of bovine tongue epithelium (NJ.Mstrain) the 50 per cent end-point dilutions in mice aged $4\frac{1}{2}$ weeks and over one year were $10^{-5.2}$ and $10^{-4.4}$ respectively.

Intranasal route. It was found with both the Ind.C and NJ.M strains that the virus dose required to infect mice 3 to 4 weeks old by the intranasal route was 200 to 1000 times that required to infect by the intracerebral route, irrespective of whether the virus strain had been passaged intradermally in guinea-pigs, or in mice by the intracerebral or intranasal routes.

In titrations of the intranasal mouse strains by the intranasal route the period of incubation tended to increase with the age of the mouse. In mice aged 3 to 4 weeks it was 5 or 6 days in 68 mice out of 86 and in mice aged 6 to 7 weeks it was 6 or 7 days in 103 mice out of 141. There was no correlation between the virus dose inoculated and the period of incubation as there was in intracerebral titrations. This is probably to be explained by the observation made by Sabin and Olitsky (1937a) that clinical signs are not observed in young mice until the infection of the anterior regions of the brain, which can be detected 48 hours after intranasal instillation of virus, has spread to other regions of the brain. In old mice this spread does not occur and a clinical infection does not develop.

Differences between the Two Strains in Infecting by the Intranasal Route

The Ind.C intranasal strain readily infected mice as old as seven weeks by the intranasal route. With the NJ.M intranasal strain the intranasal infection of mice over four weeks old was irregular. This difference between the strains could possibly be associated with the fact that the Ind.C strain originated from the 44th passage of the intracerebral mouse strain, whereas the NJ.M strain originated from the intradermal guinea-pig strain. Clarification of this point was of interest in view of a similar failure of the NJ.M egg strain to infect chick embryos at an age readily infected by the Ind.C egg strain. Material from the 29th intradermal passage in guinea-pigs of each strain was used, therefore, to initiate fresh intranasally passaged mouse strains and an experiment was

made to determine the variation with age of the susceptibility to infection by the intracerebral and intranasal routes and by ingestion and contact.

The experiments with each strain were carried out on consecutive days, the mice being divided evenly between each. The source of infective material was a pool of brains from mice of the second intranasal passage. On the day of each experiment a 1 in 10 brain suspension was prepared and dilutions were inoculated intracerebrally and intranasally into groups of 6 to 8 mice of varying age. Uninoculated mice were left in contact with the groups inoculated intranasally. Further groups were fed with bread soaked in a $10^{-0.5}$ dilution in milk, other food being withheld until all had been eaten. Each experimental group was confined to a single container.

On the twenty-first day of the experiments a pool of brains from mice which had reacted in the intranasal groups was used to test by intracerebral inoculation all the surviving mice for evidence of an acquired immunity. Mice surviving in the groups tested for their susceptibility to infection by ingestion and by contact served as controls to this test.

Intracerebral infection. The initial suspension was titrated intracerebrally in mice aged 4 and 18 weeks. The difference in titre indicated by the two age groups was not significant and the combined number of clinical infections gave a 50 per cent end-point dilution of $10^{-5.3}$ for each strain.

Intranasal infection. Details of the mice infected by intranasal instillation of virus are given in Table 2. The virus dilutions used

TABLE 2

IND.C AND NJ.M STRAINS OF THE VIRUS OF VESICULAR STOMATITIS

Comparison of the susceptibility of mice of varying ages to infection by the intranasal route

Virus strain	*Age of mice in weeks*	*Number of intracerebral ID50 instilled*									*Controls in contact*		
		200,000			6,300			200					
		Total	*C—Sc*	*N*	*Total*	*C—Sc*	*N*	*Total*	*C—Sc*	*N*	*Total*	*C—Sc*	*N*
Ind.C	48	8	1—6	0*	8	1—6	0*	6	0—2	4	4	0—0	4
	15	8	6—2	0	8	3—5	0	6	0—5	1	4	0—0	4
	7	8	8—0	0	8	7—1	0	6	1—4	1	3	0—0	3
	3½	7	6—1	0	8	8—0	0	8	6—1	1	6	2—0	2*
NJ.M	48	8	1—5	2	8	1—2	5	6	0—0	5*	4	0—0	4
	15	8	2—3	3	8	1—6	1	6	0—2	4	4	0—0	4
	7	7	1—3	3	8	6—0	2	6	0—0	6	4	0—0	4
	3½	8	6—1	1	8	6—1	1	7	1—0	6	6	0—2	4

The columns under each heading give the total number of mice inoculated; the number which developed clinical (C) or a subclinical (Sc) infection; and the number which were unaffected (N). The allocation of mice to the last two categories was determined by an intracerebral immunity test twenty-one days after intranasal instillation of virus.

*The additional mice in these groups died at the time of the intracerebral immunity test.

were $10^{-0.0}$, $10^{-1.5}$ and $10^{-3.0}$. The numbers of intracerebral ID50 instilled were therefore about 200,000, 6,300 and 200 for each strain.

In the Ind.C experiment it appeared that age had little effect on the overall incidence of infection, but the proportion of clinical infections, as compared with subclinical infections, diminished regularly with an increase in age. In the NJ.M experiment the results were less regular and all the age groups receiving the two largest doses of virus contained mice which remained free from infection, the total incidence of infection being 45/63 as compared with 63/63 in the Ind.C experiment. In the groups receiving the smallest dose the incidence was 3/25 as compared with 19/26. The Ind.C strain after only two passages in mice was therefore shown again to infect the older mice more readily than the NJ.M strain. On the fourteenth day after the intracerebral immunity test mice surviving in the intranasal groups and aged 48, 15 and 7 weeks were bled out. A pool of serum was prepared from the mice of each experiment. Both pools, with a pool of normal mouse serum as a control, were tested in mice for their neutralising activity. Each, at a dilution of 1 in 5, neutralised at least 5000 i/c ID50 of the homologous type mouse strain and less than 10 i/c ID50 of the heterologous type. A procedure such as this could, therefore, be used to prepare immune serum from mice.

Contact infection. Details of the mice left uninoculated which were in contact with mice of the same age in the intranasal groups are given in Table 2. All were in contact with mice which developed a clinical infection. All the contact mice aged, 48, 15 and 7 weeks, none of which had been in a tin in which cannibalism had occurred, were susceptible to infection in the intracerebral immunity test.

In the experiment with the Ind.C strain there were two clinical infections in the mice aged 3½ weeks as a result of contact. One of these infections occurred on the tenth day of the experiment which was four days after a mouse in the same tin was found dead with the head completely eaten. The second mouse developed a clinical infection on the sixth day of the experiment which was the day on which the intranasally infected mice ($10^{-0.0}$dilution) in the same tin commenced to develop clinical signs. It must be inferred therefore that in this case the mouse derived its infection on the zero day of the experiment, the source of virus being the recently inoculated mice. This might well be the method whereby other uninoculated mice have acquired an infection, probably through the intranasal route, when no cannibalism has been observed.

In the NJ.M experiment two of the contact mice aged 3½ weeks were infected subclinically. They were the only two which had been in a tin in which there had been cannibalism. Here again, as so often occurred, only the head had been eaten and infection by the intranasal route could have occurred readily.

Infection by ingestion. The mice given the single feed of infected

food were groups aged 48, 5½ and 3 weeks. None of the mice of the two older groups developed a clinical infection and all of them were susceptible to the subsequent intracerebral test. These groups contained five or six mice. In both experiments one mouse out of groups of eight mice aged three weeks developed clinical infection after four (Ind.C) or five (NJ.M) days. The specificity of these infections was confirmed by the inoculation of guinea-pigs. The seven surviving mice of each group were all susceptible to the intracerebral test.

Further Examples of the Incidence and Probable Causes of Subclinical Infections.

In the experiments just described the cause of the subclinical infections detected in the mice not given virus intranasally was thought to be accidental intranasal infection. This could have occurred either through the ingestion of infective food or carcasses, or licking of the excess inoculum from neighbouring mice inoculated with material containing sufficient virus to infect by the nasal route.

Several more subclinical infections were detected by the routine intracerebral immunity tests which followed most intracerebral infectivity titrations and serum neutralisation tests. An analysis of tests which followed experiments in mice aged 3 to 6 weeks shows that out of a total of 234 mice included in these tests there were 31 which were not in contact with clinically infected mice and none of them had an immunity. The remaining 203 mice were all in contact with clinically infected mice before the immunity test and can be divided into three groups.

(1) A group of 92 mice which survived the intracerebral inoculation of serum-virus mixtures. Nine of these were immune.

(2) A group of 90 mice which survived the intracerebral inoculation of virus dilutions in infectivity titrations. Eleven of these were immune, one of which could have acquired an immunity from cannibalism. Five of the remaining 10 immune mice were in a group totalling 77 mice which had been inoculated initially with less than one i/c ID50 and the other 5 were in a group totalling 13 mice which had received 1 to 8 i/c ID50. As all these 10 mice had an equal opportunity of becoming infected by contact with clinically infected mice the much greater incidence of subclinical infections in the group receiving the larger virus doses suggests that these subclinical infections were due to the initial inoculum and not to contact. They had in fact been inoculated with a virus dilution which caused a clinical infection in at least one other mouse, the total number of these infections being 24. Ten other mice in these same groups were not immune to the test. As none of these immune mice had been in contact with mice inoculated with more than 8 i/c ID50 their immunity cannot be explained by nasal infection from excess inoculum.

(3) A group of 21 mice which had been uninoculated contact controls. Four of these were immune. Two of these subclinical infections could be attributed to cannibalism and the other two to contact with mice immediately after inoculation intracerebrally with 1000 i/c ID50. There had also been four uninoculated contact controls which became clinically infected in the course of the original experiments. One of these infections could be attributed to contact with mice infected intracerebrally with 10,000 i/c ID50 and three to cannibalism.

Clear cut evidence regarding the part which cannibalism can play in the infection of adult mice was obtained with a group of female mice of varying age but all over three months. The litters of these mice were used when they were one to two weeks old for the titration of the infectivity of cattle tongue epithelium (Ind.C strain). It must remain uncertain whether any of the mothers became infected by licking the contaminated skin of their young immediately after inoculation, but out of a total of 42 females which were given an intracerebral immunity test at the conclusion of the experiments only 23 were susceptible to the test. None of these 23 mice had eaten any of their young. The litters of 6 of them had remained free from clinical infections and the litters of 17 had been infected clinically. All of the 19 females which were immune to the test had eaten some of their infected young and two of them had developed a non-fatal clinical infection prior to the immunity test.

Findings such as these give a fair warning to any worker using unweaned mice as experimental animals for the virus of vesicular stomatitis who might, in an emergency, contemplate at the conclusion of an experiment the retention of the mothers for further use as breeding stock.

The Susceptibility of Unweaned Mice to Infection by Various Routes

To determine the relative susceptibility of unweaned mice to infection by various routes, ten-fold dilutions of suspensions of infective tissue were inoculated into groups of mice aged 5 to 18 days, keeping the volume of inoculum constant at 0·03 ml.

When the intracerebral route was used mice aged 5 to 9 days were of the same susceptibility as mice aged four weeks. In an experiment with a filtrate of a suspension of cattle tongue epithelium (Ind.C strain) the 50 per cent end-point dilution in mice one week old, when inoculations were by the intracerebral and intraperitoneal routes, was $10^{-3\cdot6}$ and $\geqslant 10^{-2\cdot4}$ respectively. The intracerebral end-point was the same in mice aged 10 or 13 days but intraperitoneally a 10^{-1} dilution infected only 6 out of 11 mice 10 days old, and a 10^{0} dilution infected only 2 out of 10 mice 13 days old.

In a comparison of the intracerebral, intraperitoneal and subcutaneous routes in mice 5 and 18 days old the 50 per cent end-point

dilution of a filtrate of a suspension of cattle tongue epithelium (NJ.M strain) in mice 5 days old was $10^{-6.6}$, $10^{-4.2}$ and $10^{-3.1}$ respect tively. The intracerebral end-point was the same in mice 18 days old but only one out of twenty-four mice of this age inoculated by the other routes with 10,000 and 100,000 intracerebral ID50 developed a clinical infection. There would, therefore, appear to be a rapid change in the susceptibility of unweaned mice to infection by the intraperitoneal and subcutaneous routes during the second week of life.

In these experiments and in others with the mouse strains of virus, immunity tests detected only one subclinical infection in 36 mice which when they were 5 to 18 days old had survived the inoculation of 1000 to 100,000 i/c ID50 by the intraperitoneal and subcutaneous routes.

COTTON RATS

Wild rats, white rats and hooded rats have been shown to react with local vesicular lesions following intradermal inoculation of the metatarsal pads with the virus of vesicular stomatitis and fatal infections have been produced by intracerebral inoculation (Wagener 1931, Olitsky, *et. al.*, 1934). In the present studies it was found that adult cotton rats could be infected with this virus by the intracerebral and intranasal routes. Experience was limited to six animals about two years old.

A cotton rat which was inoculated intracerebrally with mouse passaged virus (Ind.C strain) was killed on the third day p.i. without definite clinical signs having been observed. A 1 in 1000 suspension of its brain was inoculated intracerebrally into a second cotton rat. This animal died three days later and a 1 in 10 suspension of its brain was instilled intranasally into a third cotton rat which developed clinical signs on the fifth day p.i. and was killed. The brains of these three cotton rats were titrated in mice and 1 in 10 suspensions had 50 per cent end-point dilutions of approximately 10^{-4}, 10^{-5} and 10^{-4} respectively. Young white mice were readily infected intranasally with dilutions of the last suspension and specificity of the virus was checked by cross immunity tests in guinea-pigs.

Two other cotton rats remained normal after intracerebral inoculation of a 1 in 10 suspension of mouse brain (Ind.C strain). A 1 in 5 dilution of their pooled sera collected three weeks later neutralised 10,000 i/c ID50 in a neutralisation test in mice. This indicated that they had been subclinically infected.

The sixth cotton rat was infected by intranasal instillation of virus of the 10th passage of the NJ.M guinea-pig strain. It remained normal till the fifth day p.i. when it was inactive and on the sixth day it was completely paralysed. It was killed and its brain was infective for guinea-pigs and specificity of the virus was checked.

CHICK EMBRYOS AND YOUNG CHICKENS

Growth of the Virus in Minced Chick Embryo Suspensions

Cox, Syverton and Olitsky (1933) described a method for the cultivation of the virus of vesicular stomatitis in suspensions of minced chick embryos in Tyrode's solution. From their work with strains of Indiana and New Jersey type they concluded that "this simple method suffices for the propagation of an indefinite number of culture generations". The simplicity of the method was demonstrated in the present studies during a series of sixteen passages in the minced tissue of embryos 9 to 12 days old. Passaging was initiated with virus of the Ind.C egg strain and after incubation at 37°C for three days culture fluids were diluted 1 in 100 and 0·1 ml. was added to the freshly minced tissue of one embryo suspended in about 3·0 ml. of Tyrode s solution, pH 7·5. In two consecutive passages transfer was made with a 1 in 1000 dilution and in one passage with a 1 in 100,000 dilution which approximated the limiting infective dilution for chick embryos 9 to 10 days old.

Infection of Chick Embryos by the Chorioallantoic Method

Burnet and Galloway (1934) demonstrated the high susceptibility of chick embryos to infection with the virus of vesicular stomatitis when inoculation was made by the chorioallantoic method. They worked with chick embryos aged 9 to 10 days and this host was shown to be capable of detecting virus of guinea-pig passaged strains in dilutions higher than the minimal infective dilution for guinea-pigs. Two types of response were recognised. Either the embryo died between 24 and 48 hours after inoculation without any very characteristic lesions, or it survived and changes were observed in the chorioallantoic membrane. The type of response was entirely unpredictable although the 'Indiana' strain caused the lethal response more frequently than the 'New Jersey' strain. These two strains originated from the U.S.A. and were originally recovered from field outbreaks about the year 1926. It was of some interest, therefore, to find in the present studies that the same variation of response of chick embryos 9 to 10 days old to infection with the Ind.C and NJ.M strains recovered from field outbreaks many years later was observed. To demonstrate this and other differences between these two strains they were titrated simultaneously in embryos 7, 9 and 11 days old with the results detailed in Table 3.

Irrespective of the host in which these strains had been serially passaged, it was constantly found when the eggs after inoculation were incubated at 37°C that with Ind.C virus embryos 7 to 10 days old were of equal susceptibility whereas with NJ.M virus embryos older than 8 days were more resistant to infection than those 7 days old as is also shown by the experiment in Table 3. This observation with the NJ.M strain corresponds with that made by Sigurdsson (1943) in his studies with the virus on the influence of

TABLE 3

VARIATION IN RESPONSE OF CHICK EMBRYOS

Incubated at 37°C after inoculation with virus from the 20th intradermal passage in guinea-pigs of the Ind.C and NJ.M strains

Log. of the dilution of the suspension of vesicular epithelium	*Strain and age of chick embryos when inoculated by the chorioallantoic method*						
	Ind.C				*NJ.M*		
		7 days	*9 days*	*11 days*	*7 days*	*9 days*	*11 days*
—5·7	i.	2223SS	122222	223SS	2223SS	SSSSSS	SSSS
	ii.	NNN		NNN	NNN	tNNNNN	tNNN
	iii.	oo		Do	oo	DDoooo	Dooo
—4·0	i.	112222	222222	22224	112222	22SSSS	223SS
	ii.			t		tttN	TtN
	iii.					++++	+o
—2·3	i.	111122	122222	12222	111222	122234	24SSS
	ii.					NT	TTtN
	iii						+++
—o·6	i		1111	1112		122222	1111S
	ii.						N
	iii.						+

Observations on individual embryos are detailed on lines i, ii and iii as follows :

Line i. 1, 2, 3, 4 — Embryo died 1st, 2nd, 3rd, 4th day p.i.
S — Embryo surviving on 4th day p.i.

Line ii. Thickening of chorioallantoic membrane on 3rd–4th day p.i.
N — Thickness normal
t — Slight thickening
T — Gross thickening

Line iii. On the 4th day p.i. the egg fluids and chorioallantoic membrane of each embryo surviving at the three lowest dilutions were taken and tested on guinea-pigs for infectivity. On the 9th day p.i. these tissues from the embryos surviving at the highest dilution were tested.
D — Embryo died 5 – 9 days p.i.
o — Infectivity test negative
+ — Infectivity test positive

temperature of incubation on the response of embryos 7 and 10 days old to infection. His findings are often quoted as though applicable to all strains of the virus of vesicular stomatitis whereas in fact all the data was obtained with a strain of New Jersey type.

A practical outcome of these observations is that in work with NJ.M strains only embryos 7 to 8 days old are considered suitable experimental hosts whereas with Ind.C strains embryos up to 10 days old can be used.

A study was made with the Ind.C egg strain, of the susceptibility of chick embryos older than 11 days when inoculated by the chorioallantoic method. The membranes in such cases were sometimes dropped at about the tenth day of incubation and, as in work with embryos of all ages, an opening in the shell and shell membrane, 7 x 15 mm. was made and sealed with transparent tape so that the embryos at the time of inoculation and subsequently could be viewed directly for viability by lifting the tape. In a comparative titration of virus in embryos 8, 11, 14 and 17 days old the result by

the third day p.i., based on death of the embryo, indicated that 8 and 11 day old embryos were of equal susceptibility but about 100 and 10,000 times more virus was required to kill embryos 14 and 17 days old respectively. Some of the embryos at each dilution which were surviving on the third day p.i. were killed and their tissues tested on guinea-pigs for infectivity. Virus was recovered only from embryos 14 and 17-days old. These had survived the inoculation of >100 ID50 for 8-day embryos. The remaining 14 and 17-day embryos which had received $\leqslant 10$ ID50 hatched normally but three 17-day embryos which were given >100 ID50 failed to hatch. One died six days and one seven days p.i. The other was killed on the seventh day p.i. Samples of brain tissue for infectivity tests were removed from each of these embryos by suction with a cancer inoculating needle inserted through the seared skull and virus was recovered in each case.

The same technique was adopted in further small experiments in which embryos 17 to 18 days old were inoculated by the chorioallantoic method with $\geqslant 1000$ ID50 for 8-day embryos. Virus was again recovered from the brains of three embryos. One had died on the second and one on the fourth day p.i. and one had survived in shell to the sixth day p.i. No virus was recovered from three uninoculated controls which hatched normally or from one which did not hatch. Chick embryos 19 days old were inoculated in the same way and six which hatched normally were killed and virus was recovered from a pool of their brains.

Infection of Young Chickens by the Intracerebral Route

The observations made with chick embryos 17 to 19 days old led to experiments with one day old chickens. These were inoculated intracerebrally with virus of the Ind.C egg strain, the standard volume of inoculum being 0.05 ml. Multiplication of the virus in the brain of this host was demonstrated. A series of 15 intracerebral passages was then initiated with the brain of a chick which had been inoculated intracerebrally with 10^6ID50 for 8-day chick embryos. This chick was killed in a state of collapse 24 hours p.i. and a 1 in 100 extract of its brain was used for passage. At this second passage a chick which was drowsy 24 hours p.i. was used as a donor and the same passaging procedure was continued. Donors for the 4th and 5th passages were killed on the second day p.i. when they showed a slight stagger. Thereafter donors were selected if clinical signs were observed, but usually no signs were seen and apparently normal chicks were then killed as donors 3 to 4 days p.i.

The brain tissue used at the 2nd to 5th passages contained at least 10^7ID50 per gram for embryos eight days old. Thereafter about one hundredfold less virus was usually demonstrated in the titrations carried out at each passage. At the 5th and 8th passages virus was demonstrated in 1 in 100 suspensions of the brains of chicks which died a week after inoculation, or were killed at this time when in

only fair condition. Others which remained normal were killed sixteen days p.i. and no virus was demonstrated in 1 in 5 brain suspensions.

At the 10th passage chicks aged 3, 10, 16 and 23 days were inoculated intracerebrally with a volume of brain suspension containing 100 i/c ID50 for mice. One bird at each age was killed 4, 7, 18 and 25 days later and a group of four inoculated when 14 days old was killed on the 27th day. No clinical signs were seen. The brain and serum of each bird were tested for infectivity and neutralising activity respectively. At the 4th day p.i. the brains of the chicks inoculated when 3, 16 and 23 days old were infective. Thereafter no virus was demonstrated in 1 in 5 suspensions of brain tissue. Specific neutralising activity was present in the sera of some of the birds killed 18, 25 and 27 days p.i.

Several tests were made of the specificity of the virus surviving at the 14th intracerebral passage of the Ind.C strain in chicks. Mice 5 to 6 weeks old given the virus intranasally remained normal, but were immune when subsequently tested intracerebrally with the Ind.C mouse strain. The virus infected guinea-pigs clinically by intradermal inoculation and these were shown to be immune to the Ind.C guinea-pig strain but susceptible to the NJ.M guinea-pig strain. Subclinical infection in guinea-pigs following inoculation of the virus by the subcutaneous route was demonstrated in the same way. In serum neutralisation tests in chick embryos 100,000 ID50 of the virus were neutralised by Indiana type immune guinea-pig serum but <5 ID50 by New Jersey type immune guinea-pig serum.

Young chickens were shown to be susceptible also to infection with the MEX.1 and NJ.M strains. In the MEX.1 experiments two filtrates were used. One was prepared from cattle tongue epithelium and the other from chick embryos, both hosts having been infected with virus from the first experimental passage of the field strain in cattle. The experimental results are given in Table 4. No clinical signs

TABLE 4

INFECTIVITY OF THE BRAINS OF CHICKENS

Following intracerebral inoculation with virus of the MEX.1 strain

Source of virus and age of chicks when inoculated intracerebrally with 10,000 i/c ID50 for mice		*Day p.i. when killed*				
		2nd	*3rd*	*4th*	*6th*	*20th*
Cattle vesicular epithelium	1 day	$>-4{\cdot}5$	$>-4{\cdot}5$	$-4{\cdot}0$	$-2{\cdot}0$	$\leqslant-1{\cdot}5$
	3 weeks		$-2{\cdot}5$	$-3{\cdot}0$	$\leqslant-1{\cdot}5$	$\leqslant-1{\cdot}5$
Chick embryo	1 day	$>-4{\cdot}5$	$>-4{\cdot}5$	$-4{\cdot}0$	$-4{\cdot}0$	
	3 weeks		$-3{\cdot}5$	$-3{\cdot}0$	$\leqslant-1{\cdot}5$	

Infectivity titre of a 1 in 5 suspension of three pooled brains expressed as the Log. of the approximate 50% end-point dilution infective intracerebrally for mice.

developed in any of these chicks before they were killed. A pullet fourteen weeks old was also inoculated intracerebrally with each filtrate at a dose four times that used for the younger birds. These two birds remained normal and were killed three weeks later. Their pooled sera and the pooled sera of the two groups of 1-day old chickens killed on the 20th day p.i. were tested in mice at a dilution of 1 in 100 for neutralising activity. None of the sera neutralised 20 ID_{50} whereas in the same test type specific convalescent guinea-pig serum at the same dilution neutralised 20,000 ID_{50}.

The experiments with the NJ.M strain comprised two attempts to passage the strain and further comparisons of the susceptibility of chicks of different ages. Using virus of the NJ.M guinea-pig strain (23 to 24 i/d passage) groups of chicks 3, 10 and 24 days old were inoculated intracerebrally with 10,000 i/c ID_{50} for mice. One of the eight chicks 24 days old was weak on its legs 24 hours p.i. and on the next day it was unable to stand and was killed. The brain was shown to be bacteriologically sterile and a 1 in 5 suspension was infective for mice at a 10^{-6} dilution. All the other chickens remained clinically normal and groups of two or three at each age were killed 3, 5, 7 and 21 days p.i. Each brain was tested separately for infectivity and the only one found to be infective was that of a 3-day old chick killed on the 3rd day p.i. A further passage using this brain as a source of virus failed.

The suspension of the infective brain of the 24-day old chick was then used at a dilution of 10^{-2} to inoculate intracerebrally groups of chicks 1, 3, 9 and 34 days old. All were normal when killed three days later. Tests of each brain showed that in the four age groups 5/6, 2/6, 0/4 and 0/3 respectively were infective. A third passage was successful in chicks one day old but a fourth passage failed. Reverting to the original virus source a second attempt at passaging was made using chicks 1 to 3 days old. Donors were killed on the second day p.i. without any clinical signs having been seen. The infectivity of the brain tissue was consistently low and the virus was lost after the fifth passage.

The individual infectivity tests of brains carried out in these experiments were made partly to determine whether infectivity of the brain was linked with the macroscopic changes seen in it. This was found to be so. The main change observed in these experiments and in earlier ones with the Ind.C and MEX.1 strains was a diffuse yellowish discolouration of the region surrounding the track of the inoculating needle. The next most frequent changes were a discolouration of the posterior region of the inoculated cerebrum away from the needle track or well-defined pin-head sized foci scattered on the cerebral surface. Such obvious changes as these have not been seen in the brains of mice inoculated intracerebrally with this virus.

DISCUSSION

The high infectivity of the brain tissue of cotton rats and chickens following intracerebral inoculation of the virus of vesicular stomatitis suggests that in such circumstances this tissue is the primary site of multiplication as in white mice. The comparative infectivity of other tissues was not, however, estimated. Although an acute infection was seen in a chicken inoculated intracerebrally when 24 days old the general inference from the limited number of experiments performed is that the brain becomes less favourable as a site of virus multiplication as the age of the chicken increases.

The greater difficulty experienced in maintaining the NJ.M strain in chickens by intracerebral passaging as compared with the Ind.C strain might have been linked with the factor causing the more rapid reduction in susceptibility to infection with increase in age which was observed with this strain in chick embryos. The NJ.M strain was similarly less invasive than the Ind.C strain when mice older than a month were inoculated intranasally. It is of further interest in this connection to have observed that in guinea-pigs inoculated intradermally with the NJ.M strain the incidence of secondary lesions has been found to be half that observed with the Ind.C strain (Skinner, 1957).

The warning given by Olitsky, *et. al.*, (1934) against accidental infections in mice with the virus of vesicular stomatitis virus was amply justified. Mice young enough to develop a clinical infection when inoculated by the nasal route may also become clinically infected by ingesting infected food. Excess virus contaminating mice after inoculation was also undoubtedly the source of infection in some neighbouring mice. In experimental studies with a virus which can so readily infect by the nasal route a false conclusion could be drawn from an experiment if the risk of infection by this route was not eliminated. Infection by the nasal route could occur for example in experiments investigating susceptibility to infection by inoculation of other routes if this left the skin contaminated. In the case of vesicular stomatitis virus a record of all cases of cannibalism and an application of the knowledge of the expected period of incubation following intracerebral or intranasal inoculation of virus assists in detecting accidental infections.

Lennette and Koprowski (1944) showed that in the case of many neurotropic viruses the susceptibility of mice one week old to infection by an extraneural route such as the subcutaneous or intraperitoneal was the same as the intracerebral route. In such cases an extraneural route of inoculation for serum-virus mixtures was much more sensitive for the demonstration of neutralising antibodies than the intracerebral route. The present studies indicate that in the case of the virus of vesicular stomatitis, mice one week old are much less susceptible to infection by an extraneural route than they are intracerebrally. An extraneural route cannot there-

fore be used with advantage in serum neutralisation tests. Fortunately high levels of neutralising activity against this virus can be detected when the intracerebral route is used for serum neutralisation tests in mice. This would appear to indicate a significant difference in the properties of the virus of vesicular stomatitis as compared with the virus of equine encephalomyelitis, all three types of which were investigated by Lennette and Koprowski (1944). Many other characteristics of these two viruses have been found to be very similar (Olitsky, *et. al.*, 1934).

The first hand experience obtained in these studies of the marked resistance developed by mice to infection by extraneural routes with increase in age was a potent factor in determining the choice of mice 3 weeks old rather than older mice for an experiment in 1950 in which mice were inoculated intracerebrally with the virus of foot-and-mouth disease (Skinner, 1951). These mice developed a clinical infection and it was this observation which led on to the infection of still younger mice and to the establishment of mice one week old as invaluable experimental hosts for the virus of foot-and-mouth disease (Skinner, Henderson and Brooksby, 1952; Skinner, 1953).

CONCLUSIONS

Subclinical infections with the virus of vesicular stomatitis were detected in white mice by demonstration of the presence of a solid immunity against intracerebral inoculation of 100 or more i/c ID_{50}. Such infections occurred in mice which had received intracerebrally <9 i/c ID_{50} of virus or in mice above a certain age which had received virus by the nasal route or which had in all probability been infected by this route while feeding on infective material. All the subclinical infections detected could be explained in this way and none to airborne infection from close contact with clinically infected mice.

Large doses of virus given by the intraperitoneal or subcutaneous routes to mice one to three weeks old caused a low proportion of clinical infections and surviving mice were rarely immune to an intracerebral test. With increase in age an increasing proportion of mice inoculated by the nasal route became infected subclinically and with the NJ.M strain this occurred at an earlier age than with the Ind.C strain. In mice over one year old susceptibility to infection with the NJ.M strain by the intracerebral route also appeared to diminish.

Cotton rats were found to be susceptible to infection with the virus of vesicular stomatitis.

Chick embryos 7, 8, 9 and 10 days old were equally susceptible to infection by the chorioallantoic method with the Ind.C strain of vesicular stomatitis virus, but with the NJ.M strain embryos became more resistant to infection by this method after 8 days. Virus was recovered from the brains of older chick embryos which survived

for up to a week after inoculation by the chorioallantoic method. This observation led to experiments which demonstrated transmission of the Ind.C strain for 15 passages in newly hatched chickens by serial intracerebral inoculations of infective brain tissue.

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THE VIRUS OF VESICULAR STOMATITIS IN SMALL EXPERIMENTAL HOSTS

II. Guinea-Pigs and Rabbits

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INTRODUCTION

Although white mice and chick embryos are the most economical experimental hosts for the detection of the virus of vesicular stomatitis their infection terminates in death when routine methods of inoculation are employed. Survival of the host after a clinical infection with this virus can be assured in the case of guinea-pigs and rabbits if a dermal route of inoculation is chosen as distinct from a neural route. These larger hosts are therefore of great value in certain experimental investigations which otherwise might require the use of one of the naturally susceptible domestic animals.

MATERIALS AND METHODS

The strains of vesicular stomatitis virus and the general experimental procedures were the same as those detailed in the previous paper (Skinner, 1957).

RESULTS

GUINEA-PIGS

The most convenient method available for producing in guinea-pigs a clinical infection with vesicular stomatitis virus is dermal inoculation of the metatarsal pads. This can be done by scarification or by intradermal tracking with a blunted inoculation needle. This latter method has in general supplanted the former and has been the one used in the present studies. Guinea-pig strains of the virus were passaged in this manner after being initiated with virus from cattle which in the case of the Ind.C and of the NJ.M strains came from the 5th (Pirbright) cattle passage. Subsequent passages were made either by a direct transfer of washings from a newly formed primary vesicle, obtained by introducing and withdrawing a small amount of buffered saline with the inoculation syringe, or by the inoculation of suspensions of vesicular epithelium collected from such a vesicle. Vesicular epithelium was invariably the material collected for storage and for use as a source of virus for experimental work in guinea-pigs.

Normally the guinea-pigs used weighed above 450 g. and were not older than six months. They were kept singly in bins from the time of inoculation to the time of destruction. Other details regarding the maintenance of the guinea-pigs, the technique of preparing epithelial suspensions and dilutions, and their intradermal inoculation were the same as those normally in use at this Institute in work with the virus of foot-and-mouth disease and have been described by Henderson (1949).

The Lesions in Guinea-pigs Infected Intradermally

Cotton (1926) has described the syndrome in guinea-pigs after infection by scarification of the metatarsal pads with a virus strain which was later classified as of the Indiana type. This description fits closely the observations made with the three strains (Ind.C, Mex.1 and NJ.M) used in the present studies following inoculation of the metatarsal pads by intradermal tracking.

Normally both metatarsal pads were inoculated with the same test material and the guinea-pigs were examined daily until primary lesions had developed at both sites. In many experiments the guinea-pigs had then fulfilled their purpose and were destroyed. In order to gain more information on the course of the disease, however, many guinea-pigs were retained and were examined daily or on alternate days for the development of secondary lesions on the pads of the fore-feet and on the tongue.

At the first intradermal passage of the three strains in guinea-pigs primary lesions covering the whole of the metatarsal pads developed in all guinea-pigs and the incidence of guinea-pigs developing secondary lesions was 13 out of 43 with the Ind.C strain and 3 out of 40 with the NJ.M strain. With a strain of the virus of foot-and-mouth disease one would have expected the incidence of this generalisation to have increased with further intradermal passaging in the guinea-pig, but with these two strains of the virus of vesicular stomatitis the incidence remained about the same for each throughout thirty-five intradermal passages, some of which were made in rapid succession. Secondary lesions were usually on the pad of one fore-foot only and often involved only a small area. Lesions on the tongue or lips were never observed.

Experience with the Mex.1 strain was limited to 18 guinea-pigs of which all but two developed secondary lesions. The majority of these were extensive and involved the medial aspect of the metacarpus where there is a paucity of hair. Some of the secondary lesions commenced at this unusual site.

An example of the response of guinea-pigs on each metatarsal pad to the intradermal inoculation of five-fold dilutions of two virus suspensions is given in Table 1. If all the inoculated sites were of equal susceptibility and if the reaction of one pad did not influence the reaction of the other a large group of guinea-pigs inoculated with the 50 per cent positive end-point dilution would be expected to be made up by three groups, namely, a group containing 50 per cent of the total in which primary lesions had appeared on one foot only, and two groups each containing 25 per cent of the total in which primary lesions had appeared on both feet or had not appeared at all. Such a response occurred in these two experiments. It is evident, therefore, that with these strains of the virus of vesicular stomatitis it is possible to count each primary site of reaction as a positive observation for the calculation of a 50 per cent positive end-point dilution from the result of

TABLE 1

INTRADERMAL INOCULATION OF GUINEA-PIGS WITH MINIMUM INFECTIVE DILUTIONS OF VIRUS

Response in groups of 24 guinea-pigs inoculated on both metatarsal pads with each dilution of the virus suspension	*Virus strain and log. of the dilution of a 1 in 25 suspension of guinea-pig pad epithelium which was inoculated.*					
	Ind.C			*NJ.M*		
	2·1	*2·8*	*3·5*	*2·4*	*3·1*	*3·8*
Total number of primary lesions	48	45	25	36	13	2
Percentage of positive sites ..	100	94	52	75	27	4
Percentage of guinea-pigs at each dilution which developed primary lesions on neither (*0*), one (*1*), or two (*2*) pads						
0	0	4	29	12	59	96
1	0	4	46	25	29	0
2	100	92	25	63	12	4

an infectivity titration. In the case of guinea-pigs inoculated in the same way with the virus of foot-and-mouth disease it is not possible to obtain more than one observation from each animal (Henderson, 1949). Here the issue is largely complicated by the short interval at which secondary lesions may appear after the development of a primary lesion, thus making it difficult to differentiate between primary and secondary lesions on the inoculated pads.

This difficulty did not appear to arise in the case of the strains of vesicular stomatitis virus under consideration. Information on the time of appearance of primary and secondary lesions has been extracted from the results of 43 infectivity titrations in which serial five or ten-fold dilutions were each inoculated into both metatarsal pads of three or more guinea-pigs. The data on primary lesions is given in Table 2. It will be seen that the interval after inoculation

TABLE 2

VIRUS INOCULATED INTRADERMALLY

The relationship between inoculation into guinea-pigs and the time of appearance of the primary lesions. Data from intradermal infectivity titrations of the Ind.C guinea-pig strain.

Approximate number of ID_{50} inoculated intradermally into each hind pad.	*Percentage of total number of primary lesions which appeared on the 2nd to 6th day after inoculation.*				*Percentage of guinea-pigs developing two primary lesions in which these lesions appeared* (i) *simultaneously* (ii) *on consecutive days*	
	Total	*2nd day* %	*3rd day* %	*4th–6th day* %	(i)	(ii)
<1	29	31	38	31	—	—
1—10	314	56	35	9	75	25
20—500	176	85	14	1	87	13
>1000	All primary lesions appeared 1st day				100	0

at which the first primary lesion develops is related to the amount of virus inoculated, but when two primary lesions developed they always appeared on the same day or on consecutive days. The incidence of secondary lesions was the same whether one or two

primary lesions developed. Out of a total of 250 guinea-pigs infected intradermally with the Ind.C guinea-pig strain and examined daily after the appearance of primary lesions, 67 (27 per cent) developed secondary lesions. None of these guinea-pigs developed secondary lesions within two days of the appearance of the first primary lesion. The percentage of these guinea-pigs in which the secondary lesions were first seen on the 3rd, 4th, 5th, 6th, 7th and 8th to 9th days respectively after the appearance of the first primary lesion was 4, 16, 21, 42, 14 and 3 respectively. No secondary lesions appeared at a later interval. Similar observations regarding the time of development of primary and secondary lesions were made with the NJ.M and Mex.1 strains.

As the material inoculated intradermally into guinea-pigs does not require to be bacteriologically sterile a great advantage which this animal possesses over mice or chick embryos as an experimental host is the certainty of infection which is indicated by the primary vesicle. When mice or chick embryos are used as experimental hosts the specificity of deaths requires sometimes to be checked. The guinea-pig is ideal for this purpose for in the case of a specific death, the virus content of mouse brain or egg tissue is such that a 1 in 10 suspension of either, or, for greater convenience, undiluted egg amniotic fluid, will produce a full primary lesion in the guinea-pig within 20 hours.

The Thermal Reaction in Guinea-pigs Infected Intradermally with the Virus of Vesicular Stomatitis and of Foot-and-mouth Disease

Differentiating characteristics of the clinical syndrome in guinea-pigs following infection with the virus of vesicular stomatitis or of foot-and-mouth disease had been sought by earlier workers, but the conclusion had always been that these infections could not be differentiated in this way (Cotton, 1926; Olitsky, Traum and Schoening, 1928). This is undoubtedly true if observations are confined to the sites of vesicle formation. Cotton (1926) had, however, observed that in guinea-pigs infected with the virus of vesicular stomatitis by scarification of the metatarsal pads, there was a marked rise in body temperature at the time of the earliest stages of formation of the primary vesicles. This was followed by a fall to normal after the formation of the vesicles and a further rise at the time of appearance of the secondary lesions. In guinea-pigs infected in a similar manner with the virus of foot-and-mouth disease it has been stated that there was no rise in body temperature until the appearance of secondary lesions (Foot-and-mouth Disease Research Committee, 1925).

The observations recorded by Manthei and Eichhorn (1941) indicated how regular and marked the thermal reaction was in guinea-pigs 24 hours after they had been inoculated intradermally with the virus of vesicular stomatitis and it was evident that a further investigation might enable the conclusion to be drawn that the time

at which this reaction occurred was a characteristic of this virus and not of the virus of foot-and-mouth disease.

The procedure first adopted was to record the rectal temperature of the guinea-pigs once daily for a few days prior to intradermal inoculation of virus and then twice daily at 10 to 11 a.m. and 3 to 4 p.m. during the period of formation of lesions. The recordings prior to inoculation failed to indicate that there was any incidence of concurrent infection in the guinea-pigs which caused abnormal rises in body temperature. For the latter half of the investigations temperature recording commenced after inoculation of guinea-pigs used for the titration of virus. Guinea-pigs inoculated with the highest and lowest dilutions were selected so that the former group was thus composed largely of animals which did not develop lesions and the latter group of animals which did. The control temperature readings obtained from the non-reacting guinea-pigs again remained steady. It was concluded, therefore, that abnormal rises in temperature when they did occur in guinea-pigs infected with the viruses were not likely to be the result of concurrent infections or the inoculation and examination procedures *per se*. These sources yielded the data presented in Table 3.

In discussing the results it will be convenient to consider that the conditions during the experiments were such that a morning temperature reading above 102.5°F, and an afternoon temperature reading above 103·5°F, each indicated a significant rise in body temperature.

With all the strains of vesicular stomatitis which were investigated it was found that the majority of guinea-pigs commenced to show a significant rise in body temperature when the first traces of a primary lesion had been present for about three hours. The rise continued sharply and reached its peak about three hours later when the pad epithelium over the area bounded by the inoculation tracks was completely underrun and was raised at its centre by the pressure of underlying fluid. From this point onward the vesicle lost its appearance of distension as the surrounding epithelium became underrun and during this time the temperature fell back to normal having remained above the level of significance for not longer than a total of about 20 hours. This was found to be a very consistent sequence of events.

In the guinea-pigs infected with the virus of foot-and-mouth disease the primary lesions often took longer than six hours to develop fully from the time the first trace of vesicular formation could be seen, but throughout this time no significant rise of body temperature occurred. With the guinea-pig strains of virus secondary lesions appeared in the majority of cases on the day following the eruption of the primary lesions and just prior to their appearance there was usually a significant rise in temperature which was generally proportional to the severity of the secondary lesions and which often persisted for over 24 hours. With the cattle strains a smaller

TABLE 3

MAXIMUM RECTAL TEMPERATURE RECORDS IN GUINEA-PIGS

The maximum rectal temperature at the time of formation of primary lesions following intradermal inoculation of both metatarsal pads with strains of the viruses of vesicular stomatitis and foot-and-mouth-disease, and the range of rectal temperatures recorded in non-infected control guinea-pigs.

Virus and strains producing the primary lesions		*Total number of rectal temperature readings/and guinea-pigs from which percentage is calculated.*	*Incidence of the highest rectal temperature recorded in each infected guinea-pig during formation of primary lesions and the temperature range of non-infected guinea-pigs, both expressed as a percentage of the total number of readings.*							
			99·1 to 100·0°F %	*100·1 to 101·0°F* %	*100·1 to 102·0°F* %	*102·1 to 103·0°F* %	*103·1 to 103·5°F* %	*103·6 to 104·0°F* %	*104·1 to 105·0°F* %	*105·1 to 106·5°F* %
Vesicular stomatitis	Strains passaged intradermally in guinea-pigs Ind.C.	45/45	0	0	0	2	4	7	69	18
	NJ.M	63/63	0	0	1	6	10	21	51	11
	Strains passaged intradermally in cattle Ind.C	37/37	0	0	3	14	8	8	62	5
	NJ.M	35/35	2	0	0	11	9	26	43	9
	Summary of totals	180/180		1		8	8		83	
Foot-and-mouth disease	Strains passaged intradermally >1000 times in g.p.s. (1, GB&GC)	56/56	0	4	16	30	25	16	9	0
	Strains passaged intradermally 20–24 times in g.p.s (Rho.1 & Bec. 1)	37/37	0	3	24	60	5	8	0	0
	Strains passaged intradermally in cattle (119, 149, 733 and U3)	42/42	0	2	36	53	7	2	0	0
	Summary of totals	135/135		27		45	13		15	
Non-infected control guinea-pigs	Paired readings taken from the same guinea-pigs a.m.	139/95	12	47	40	1	0	0	0	0
	and p.m. p.m.	139/95	4	23	47	23	3	0	0	0
	All readings pooled	692/185	8	34	45	12	1	0	0	0
	Summary of p.m. readings for comparison with the infected guinea-pigs	139/95		74		23	3		0	

proportion of the guinea-pigs developed secondary lesions as early as the day following the appearance of the primary lesions and the rise in body temperature at the time of this generalisation was often slight and did not appear to be a consistent feature.

Table 3 has for the sake of simplicity been compiled from the highest temperature recorded in each guinea-pig whether it was taken a.m. or p.m. Consequently it is possible to say with certainty that only those temperatures above 103·5°F indicate a significant rise. Of the 180 guinea-pigs infected with the virus of vesicular stomatitis 83 per cent had temperatures above this level. Almost all the remaining guinea-pigs had the peak of development of their primary lesions between the p.m. and a.m. temperature recordings and the a.m. recording after the formation of the lesions was above 102·5°F. Had the readings been taken more frequently it is believed that almost all would have shown a temperature reading above 104°F.

Out of the 135 guinea-pigs infected with strains of foot-and-mouth disease virus only 20, namely 15 per cent, showed a rise of body temperature above 103·5°F prior to the formation of secondary lesions. Thirteen of these 20 guinea-pigs were showing severe secondary lesions within 18 hours of the time of the selected maximum temperature which must therefore have been recorded within 2 to 12 hours of the appearance of secondary lesions. Rises in body temperature to above 104°F occurred only in guinea-pigs infected with the well-adapted guinea-pig strains.

This evidence justifies the assertion that a characteristic of the virus of vesicular stomatitis appears to be that infection of guinea-pigs with cattle or guinea-pig passaged virus by intradermal inoculation of the metatarsal pads is followed by a rise in body temperature of 2 to 3°F within a few hours of the first appearance of vesicle formation. In the case of similar infections with the virus of foot-and-mouth disease there is no significant rise until many hours after the primary lesion is fully formed.

These facts were known by earlier workers in each sphere of research. Their significance as specific characteristics of each virus was probably not recognised owing to the syndromes being complicated by concurrent bacterial infections etc. With the present guinea-pig stock from the Pirbright colony such complications were absent and enabled this distinct difference in the syndromes to be recognised.

Development of Immunity Following Infection by the Intradermal Route

A series of tests was carried out with the Ind.C and NJ.M guinea-pig strains of vesicular stomatitis virus to determine how soon after the eruption of a primary lesion on a single metatarsal pad inoculated intradermally a sufficient immunity had developed to prevent the eruption of a lesion on the other pad when it was inoculated with at least 1000 ID_{50} of the homologous

strain. This was found to occur at about the 7th day with both strains. Up to the third day after the appearance of the primary lesion on the first pad, inoculation of the second pad was followed by the appearance of a severe local lesion. From the 4th to the 7th day an increasing number of guinea-pigs failed to develop any lesion on the second pad, the proportion for each of these days being 2/10, 5/6, 23/24, 10/10 for the Ind.C strain. With the NJ.M strain the proportions for these days were 2/4, 9/13, 6/9, 5/5, and for the 8th to 9th days 17/17.

It was evident that this finding could be applied to the design of type immunity tests in guinea-pigs of unknown virus strains and to corroborate this such a test was carried out with the Ind.C and NJ.M cattle strains. Large groups of guinea-pigs were inoculated intradermally on the left metatarsal pad with 1 in 5 suspensions of cattle tongue epithelium. Local lesions developed in 24 to 48 hours and some guinea-pigs subsequently developed secondary lesions. If these occurred on the right metatarsal pad the guinea-pig was eliminated from the experiment. At the 6th, and again at the 9th to 10th day after the eruption of the primary lesions, groups of 8 to 11 guinea-pigs were selected for the intradermal test on the right metatarsal pad with the Ind.C and NJ.M guinea-pig strains, using a 1 in 10 suspension of pad epithelium in each case. The results of these tests are given in Table 4. In all cases the end result was reached on the day following the type test. Thus the test on the sixth day identified the type of the two bovine strains on the eighth day after they had been used to infect the guinea-pigs.

With the normal technique of infecting guinea-pigs with unknown strains on both pads, and waiting for the lesions to heal, it

TABLE 4

RAPID TYPE TESTING IN GUINEA-PIGS OF CATTLE STRAINS

Cattle strains used initially to infect each guinea-pig on the left metatarsal pad.		*Ind.C*				*NJ.M*			
Interval between appearance of primary lesion and type immunity test		6 days		9—10 days		6 days		9—10 days	
Guinea-pig strains used in immunity test to inoculate right metatarsal pad		Ind.C	NJ.M	Ind.C	NJ.M	Ind.C	NJ.M	Ind.C	NJ.M
Result of type immunity test. Number of guinea-pigs	Completely immune (No lesion)	7	0	11	0	0	9	0	10
	Partially immune (Mild lesion)	1	0	0	0	0	1	0	0
	Not immune (Severe lesion)	0	10	0	9	10	0	9	0

would have been at least a week later before the type test could have been carried out. If the more rapid test was to be adopted as a routine it would have to be borne in mind that 100 per cent immunity to a homologous type guinea-pig strain could not be expected earlier than the 7th to 8th day after appearance of the primary lesion produced by the virus being typed. Up to the 10th day at least there would be present the risk of contaminating the inoculation needle used for the challenge virus with virus in the apparently normal pad of the convalescent guinea-pig, for secondary lesions have appeared as late as the 9th day. Subsequent inoculations with the contaminated needle of guinea-pigs within a group immunised with virus from the same source would not matter, but a fresh syringe would have to be used for another group inoculated with the same challenge virus. If these precautions are observed there would appear to be no reason why type tests in guinea-pigs of vesicular stomatitis virus should not take the form of the tests in Table 4. The readiness with which generalised lesions would occur on the second pad precludes this form of test for the virus of foot-and-mouth disease.

The duration of the immunity against intradermal inoculation of the homologous type virus has not been fully investigated. It was found however that some guinea-pigs developed primary lesions when tested four to five months after the initial infection. Others still retained a complete immunity after this interval.

Neutralising Activity of Serum Following Infection by the Intradermal Route

Some studies have been made on the neutralising activity of convalescent guinea-pig serum collected from a group of 57 guinea-pigs all infected at the same time by intradermal inoculation of both pads with the Ind.C guinea-pig strain. Groups of at least three guinea-pigs were bled out at intervals after infection commencing at the 7th day after the appearance of the primary lesion. A pooled serum at each interval was prepared. Neutralisation tests were carried out in eggs with virus of the Ind.C egg strain. In the first test the number of ID_{50} neutralised by a 1 in 4 dilution of pooled serum collected 7, 10, 14, 18 and 22 to 26 days after the appearance of primary lesions was log. 2·8, 2·7, 3·7, 4·2 and 4·9 respectively. In a second test pooled sera collected at adjacent intervals were again pooled and the number of ID_{50} neutralised by a 1 in 10 dilution of serum collected at 7 to 10, 14 to 18, 22 to 31, 36 to 47 days, and 9 to 12, 15 to 19, 23 and 45 weeks was log. 3·1, 3·8, 4·4, 3·7, 3·7, 4·1, 4·0 and 4·5 respectively.

These tests indicated that neutralising activity may be present in the serum seven days after the appearance of the primary lesion and that when it had reached a peak at about the third week there may be no marked fall in activity throughout the following 10 months.

Comparison of Small and Medium-sized Guinea-pigs for Intradermal Infectivity Titrations

On six occasions when titrations of the Ind.C and NJ.M guinea-pig strains have been done on guinea-pigs of standard weight—viz. about 450 g. or over—the same dilutions have been used to inoculate an equal number of younger guinea-pigs weighing only 250 to 350 g. The inoculation tracks in these younger guinea-pigs were about half the length of those in the older guinea-pigs and the area of dermal tissue exposed to virus was thus proportionally smaller. In four of the titrations the 50 per cent positive end-point dilution was the same in both groups. In the remaining two titrations it was log. 0·7 lower in the younger guinea-pigs. The smaller guinea-pig did not, therefore, show any great difference from the larger guinea-pig in its susceptibility to intradermal inoculation of virus.

This information is of value in assessing the results of experiments such as those described later in which very young guinea-pigs were infected by the intranasal route. It has not led to the use of the younger guinea-pig for routine experimental work.

Subclinical Infections Induced by Inoculation of Virus by Routes other than the Intradermal

Wagener (1931a) investigated a wide range of routes other than dermal inoculation of the metatarsal pads for the infection of guinea-pigs with vesicular stomatitis virus. He found that virus introduced by these routes (intramuscular, subcutaneous, intraperitoneal, intranasal etc.) rarely caused the appearance of dermal lesions, but almost all of the guinea-pigs acquired a solid immunity against an intradermal inoculation of virus given at a later date. A smaller proportion of subclinical infections followed the drinking of infected water or contact with infected guinea-pigs. As a result of these observations he laid stress on the importance of keeping experimental guinea-pigs in separate bins if subclinical infections were to be avoided.

Sabin and Olitsky (1938) when using cerebrally passaged mouse strains of Indiana and New Jersey type found that the minimal infective dose for mice aged three weeks or more and for guinea-pigs aged 1 to 2 weeks was the same when the intracerebral route of inoculation was used. Guinea-pigs two months old required a dose ten times greater. Mice two weeks old were readily infected by the intranasal route, but guinea-pigs one week old or older were difficult to infect clinically in this way. The clinical signs observed in guinea-pigs infected by these two routes were a flaccid paralysis of the posterior extremities or an encephalitis. No dermal lesions were recorded. Since direct inoculation of the sciatic nerve was sometimes followed by an ascending myelitis it was concluded that the reason for no involvement of the central nervous system occurring after intramuscular or intradermal inoculation of the virus was that the terminal nerve endings formed a barrier to the progression of the virus. No attempt was made in this work to determine whether subclinical infections had occurred in the guinea-pigs.

In the present studies the susceptibility of guinea-pigs to infection with the Ind.C and NJ.M intradermally passaged guinea-pig strains when given by the intranasal route was investigated. Pad suspensions containing 1000 intradermal (i/d)ID50 for guinea-pigs were given at a dose of 0·1 ml. With the Ind.C strain five guinea-pigs 9 weeks old and seven guinea-pigs 3 to 4 weeks old remained normal following the inoculation. With the NJ.M strain six guinea-pigs 4 weeks old remained normal, but two out of four guinea-pigs 2 to 3 weeks old developed slight lesions on the pads of the feet. All the non-reacting guinea-pigs were found to be immune to the homologous virus strain when tested later intradermally. In a further experiment with the NJ.M strain three groups of five guinea-pigs 3 months old were inoculated intranasally with 0·1 ml. of pad suspensions containing 1, 10 and 100 i/d ID50. The number of guinea-pigs which developed dermal lesions or became infected subclinically or acquired no immunity was 0/2/3, 0/4/1 and 1/4/0 for these three doses respectively. Thus very small doses of virus, as measured by the intradermal inoculation of guinea-pigs, can set up a subclinical infection if instilled intranasally. It is known, however, that virus can be detected readily beyond the limiting infective dilution intradermally in guinea-pigs by the inoculation of chick embryos.

In a small experiment with the Ind.C guinea-pig strain an attempt was made to determine whether a thermal reaction occurred in guinea-pigs inoculated by one of four routes namely intranasally, subcutaneously, intramascularly and intraperitoneally with 0·2 ml. of a dilution of a filtrate of pad epithelium containing 200 i/d ID50. In addition, two guinea-pigs were inoculated intradermally and were placed in contact with two uninoculated guinea-pigs in a cylindrical open bin 38 cm. diameter and 46 cm. high. Rectal temperatures of all the guinea-pigs were recorded prior to exposure to infection and in the morning and afternoon for three days afterwards. Thereafter recordings were made in the morning only and until the eighth day. On the 12th day all the guinea-pigs were given a severe intradermal test for immunity. The results were as follows.

Intradermal and contact groups (*2 guinea-pigs in each*). The guinea-pigs inoculated intradermally commenced to show a rise in temperature on the afternoon of the first day p.i. but primary lesions were not seen until the following morning. The temperatures of both guinea-pigs did not return completely to normal until the 4th day p.i. On the 6th day p.i. both developed secondary lesions with some extension on the 7th day. The two guinea-pigs in contact maintained normal temperatures throughout the experiment but on retest one was completely immune and the other developed only mild primary lesions.

Subcutaneous, intramuscular and intraperitoneal groups (*2 guinea-pigs in each group*). None of these six guinea-pigs developed lesions but five of them were immune on retest. The sixth guinea-pig was in

the intramuscular group and developed mild lesions on retest. This guinea-pig and its partner had both shown significant rises in temperature during the day following inoculation but not thereafter. No significant rises in temperature were recorded in the guinea-pigs of the other two groups.

Intranasal group (*5 guinea-pigs*). Three guinea-pigs showed no rise in temperature. One showed a slight rise during the second day p.i. These four guinea-pigs developed no dermal lesions but were all immune on retest. The fifth guinea-pig had a temperature of 104·8 on the afternoon of the first day p.i. and 102·8 on the morning of the second day. Subsequent temperatures were normal. On the fifth day p.i. a lesion commenced to form on one hind pad and developed to a full lesion by the 7th day. The thermal reaction in this guinea-pig was thus very similar to that seen following intradermal inoculation and at the time of formation of the primary lesion. Also the lesion which formed later appeared at an interval corresponding with the appearance of secondary lesions following a primary dermal lesion.

It is evident that the interest of this experiment lies in the fact that a severe thermal reaction does not appear to be a consistent accompaniment of a subclinical infection and in the confirmation that an immunity may be acquired by close contact with infected animals. In subclinical infections such as the latter the route of entry of the virus is still unknown.

It has been generally recognised that some guinea-pigs which fail to develop primary lesions following intradermal inoculation of a limiting infective dose of vesicular stomatitis virus may be found to be solidly immune when retested intradermally with an adequate virus dose. Such instances have been observed in the present studies when non-reactors have been tested, but they were not of common occurrence and did not occur in guinea-pigs inoculated intradermally with dilutions ten-fold or more higher than the limiting infective dilution. There was, therefore, no evidence to suggest that these subclinical infections arose from virus conveyed in some way from infected guinea-pigs in the same room.

Serum Neutralisation Tests using Guinea-pigs, Chick Embryos and White Mice

Examples of the high neutralisation indices of the sera of convalescent guinea-pigs and rabbits as indicated by neutralisation tests using chick embryos and white mice are given in this paper. Equally high neutralisation indices are obtained if guinea-pigs are used. Full details of the techniques for such tests will not be recorded here because it has been found that the standard methods adopted in virus research are applicable to the virus of vesicular stomatitis. Thus, in a test for typing a virus strain normal serum and two specific type immune sera are each mixed after inactivation at 56°C for 30 minutes with an equal quantity of a series of ten-fold

dilutions of the virus suspension and the mixtures left at room temperature in the dark for about an hour. These can then be inoculated into guinea-pigs by the intradermal route, into chick embryos by the chorioallantoic method or into mice by the intracerebral route.

Several factors which might influence the result of a neutralisation test designed to detect small differences in neutralising activity have been investigated. These experiments yielded no unexpected results and do not warrant a detailed description. It was found that the results obtained were the same whether serum-virus mixtures kept at room temperature or at 37°C were inoculated after contact for 30 minutes or for 55 to 80 minutes. Significantly less neutralisation was detected, however, when mixtures kept at 5°C, room temperature or 37°C were inoculated after contact for only two or five minutes as compared with 30 minutes.

For a critical comparison of the neutralising activity of two sera it might be necessary to mix dilutions of the test sera with a single dilution of virus. In such tests it was found that the result was the same whether the dilutions of serum were prepared in 0·15M NaCl buffered at pH 7·6 with 0·005M phosphate salts or in undiluted normal serum. Varying degrees of agitation of the mixtures did not affect the results when serum dilutions were prepared in normal serum. Samples of immune serum stored at 4°C for a year undried and dried were found to possess the same neutralising activity.

Confidence in the reliability of these serum neutralisation tests was obtained from tests in which the neutralising activity of artificially prepared sera of known antibody content was assayed. These sera were a standard convalescent guinea-pig serum and $10^{-0.5}$, $10^{-1.0}$ and $10^{-1.5}$ dilutions (x 3·2-fold series) of it in normal guinea-pig serum. Tests were carried out on separate occasions with guinea-pigs, chick embryos and white mice as experimental hosts. Mixtures of 3·2-fold dilutions in buffered saline of each test serum and a single virus suspension were inoculated into sufficient hosts to give 5 to 8 observations for each mixture for the calculation of the serum dilution at the 50 per cent end-point. The results are given in Table 5 and indicate within each test that a constant amount of antibody

TABLE 5
NEUTRALISING ACTIVITY OF SERA WITH KNOWN VARIATIONS IN CONCENTRATION OF ANTIBODY

Log. of dilution of original immune serum made in normal serum to give the test serum.	*Log. of 50% end point dilution of test serum in first column which neutralised the ID50 indicated in tests using guinea-pigs, chick embryos and mice. Serum dilution prepared in buffered saline.*		
	Guinea-pigs ($10^{3.3}$ *ID50*)	*Chick embryos* ($10^{3.0}$ *ID50*)	*Mice* ($10^{1.9}$ *ID50*)
−0·0	−3·7	−3·3	−2·7
−0·5	−3·0	−2·5	−2·3
−1·0	−2·8	−2·0	No test
−1·5	−2·2	No test	No test

was present in the mixtures at the 50 per cent end-points. Thus in the guinea-pig test the dilutions of the original immune serum added to the 50 per cent end-point mixture would have been $10^{-3.7}$, $10^{-3.5}$, $10^{-3.8}$ and $10^{-3.7}$. The variation of the total serum concentration in the final mixtures by a factor of $10^{1.5}$ did not therefore appear to influence the antigen-antibody reaction and tests designed in this way were sufficiently sensitive to detect 3·2-fold differences in concentration of neutralising antibody.

RABBITS

Routes of inoculation by which earlier workers succeeded in producing a clinical infection in rabbits with the virus of vesicular stomatitis were scarification of the cornea or of the inner surface of the lips, and inoculation of the brain. Vesicles formed at the sites of scarification and nervous signs which developed a week after intracerebral inoculation progressed to a complete flaccid paralysis of the hind limbs (Olitsky 1927; Olitsky, Cox and Syverton 1934). Wagener (1931b), however, did not find the buccal or corneal epithelial routes satisfactory and it therefore seemed of some interest to determine which was the most satisfactory route for the production of a clinical infection, for the rabbit might prove to be a valuable host for certain aspects of experimental work, for example in imunity studies involving repeated sampling of blood.

From the outset it was evident that inoculation of the papillated epithelium of the tongue by tracking with a fine blunted inoculation needle would be the most practical one for use as a routine. This method was found to be entirely satisfactory for local lesions were produced readily by strains of virus from many variable sources. These sources were cattle tongue epithelium (27 infections), guinea-pig pad epithelium (44 infections), chick embryo tissues (19 infections) and rabbit tongue epithelium (46 infections). These infections were divided almost equally between the Ind.C and NJ.M strains and virus from all these sources appeared equally capable of inducing an infection.

Other routes of inoculation were not investigated in more than a cursory fashion and in the little work which was done it was found that inoculation of large doses of Ind.C strain virus by scarification of the cornea (6 rabbits) or by the intracerebral route (4 rabbits), or intracutaneously into the hairy skin (4 rabbits) in at least two experiments for each route, produced only a subclinical infection or no infection at all as indicated by subsequent immunity or susceptibility to infection by inoculation of the tongue epithelium.

Course of Infection Following Inoculation of the Tongue Epithelium

Inoculation technique. By intravenous inoculation of Nembutal (Abbott) a deep narcosis was induced in groups of 5 to 10 rabbits at a time. The virus suspensions were then inoculated with a fine blunted needle (0·4 mm. : 20 mm.) along six parallel tracks nearly 2 cm. long, made in the tongue

epithelium while the tongue was held in the fingers by its tip and withdrawn from the buccal cavity.

Two methods of preparing and inoculating a single track have been tried. In the first method the inoculating needle was not allowed to break out from the epithelium at the distal end of the track and with the needle half withdrawn inoculation was then made into the blind track under pressure. If this was done slowly and cautiously a bleb about three times the width of the original track formed throughout most of its length and collapsed immediately the needle was completely withdrawn. In the second method the inoculating needle was directed so that it broke through the epithelial surface at the distal end of the track which was then inoculated continuously whilst the needle was withdrawn. When both methods were compared on the same tongue it was found that tracks inoculated by the first method were the site of more severe primary lesions than those inoculated by the second method. This was due no doubt to the increased area of epithelial tissue exposed to the virus by that method.

To examine the tongue for lesions an assistant extended the animal across his thigh with the chest uppermost and head held back. The anterior portion of the tongue was then gripped with dressing forceps and gently withdrawn until the whole of its surface could be examined. Dental jaw openers were found valuable aids for this manipulation and prevented contamination of the hands with virus. Freshly sterilised instruments were used for each animal.

When rabbits were used for the titration of virus suspensions ten-fold dilutions were prepared and each was inoculated into at least five rabbits, each rabbit being used to give one observation. On three occasions samples of the same dilutions of tissue suspension being tested in rabbits were inoculated into groups of 5 to 7 guinea-pigs by intradermal inoculation of both metatarsal pads. In each case the 50 per cent positive end-point dilution was almost identical for each host.

Comparisons were made between rabbits of the Chinchilla, Dutch and Blue breeds and these showed no variation in susceptibility. It was however of some advantage to work with a rabbit with a large tongue and of these three breeds Chincillas were for this reason preferred, the tongue at five months of age (1800 g. body weight) being of adequate size. Susceptibility to infection at this age was found to be the same as that of rabbits two years old or more.

Primary and secondary lesions. If tracking of the tongue epithelium was carried out with buffered normal saline as the inoculum the sites of the tracks 20 hours later showed clearly as 'white lines' viz. a blanching of the epithelium immediately over the tunnelled track. In some animals this epithelium was already sloughed off at many points leaving a shallow furrow. By the second day after inoculation the underrun epithelium had always been exfoliated completely.

Following inoculation of virus suspensions the specific primary lesions invariably developed from the site of a 'white line' and with suspensions containing only 1 to 100 m.i.d. the tongue 20 hours after inoculation often showed only a trace of any extension of the

underrunning of the epithelium from these lines. During the 24 hours that followed progression of this underrunning was completed and it then involved the whole or only a very small part of the inoculated area. With more concentrated suspensions of virus this final stage of the primary lesion was reached by the 24th hour p.i. The underrun epithelium did not become distended by fluid and was usually sloughed off within one or two days. The underlying epithelium was healthy, but irregularities in the formation of papillae usually enabled the site of a former lesion to be detected for a variable time depending on its original severity.

Thirty-two out of 73 rabbits which were examined for at least a week after the appearance of a primary lesion developed secondary lesions on the tongue, in all cases on the 5th to 7th day after inoculation. There was, therefore, a period of at least two days between signs of the primary and secondary lesions during which the tongue epithelium appeared perfectly healthy. The secondary lesions extended more deeply into the epithelial layers than the majority of primary lesions and were sometimes true vesicles distended with fluid. Epithelium collected from a secondary lesion of a rabbit infected with virus of the NJ.M guinea-pig strain produced very severe lesions when titrated intradermally on guinea-pigs, a 1 in 10 suspension having a limiting infective dilution of 10^{-4}. It was frequently observed that a severe primary lesion was not followed by a secondary lesion, although ample healthy tongue epithelium was present, and that the secondary lesion tended to be most severe when the primary lesion had been weak. With lack of experience it would be easy to miss a small primary lesion and only to observe the secondary lesion.

Inappetance usually accompanied the formation of a primary lesion and often appeared to be more marked, and to last longer, than would be accounted for by the visible lesions alone and was possibly an indication of a more systemic infection. A small attempt was made to investigate this matter further by studying the thermal reaction and carrying out tests for a viraemia.

Thermal reaction. Under the particular conditions in which the investigations were carried out it was found that the individual temperature recordings in normal rabbits often remained remarkably constant from day to day and that there was often no diurnal variation. When the rectal temperature was followed in two experiments for several days before and after intradermal inoculation of the tongue with vesicular stomatitis virus in a total of 25 rabbits, it was noted that in the eleven rabbits which developed a severe primary lesion—i.e. the lesion extended over more than half of the tongue—there was a rise in temperature of 1 to 2°F during the development of this lesion and then a return to normal. This small rise could definitely be classified as significant. With less severe primary lesions a smaller rise or no rise in temperature was noted.

Tests for a viraemia. In a single experiment with four rabbits,

two of which developed severe and two moderate primary lesions at the 20th to 24th hour p.i., samples of blood were taken at the 20th, 24th, 44th, 48th and 68th hours after inoculation of the tongue and were citrated and tested immediately for infectivity by the intradermal inoculation of guinea-pigs. In none of the 20 samples was any virus detected.

Immunity Following a Clinical Infection and Neutralising Activity of the Serum

A total of eleven rabbits from several experiments were tested for immunity by intradermal inoculation of the tongue with a large dose of homologous type virus 2 to 3 weeks after a clinical infection. None of them developed lesions and controls were satisfactory. Nine rabbits clinically infected with the NJ.M guinea-pig strain were fully susceptible to the Ind.C guinea-pig strain when tested with it five weeks later.

Seven pools of rabbit serum were prepared during the course of experiments. They were filtered and stored at 4°C. After being stored for $2\frac{1}{2}$ to $3\frac{1}{2}$ years their neutralising activity was assayed by tests in mice with a control pool of normal rabbit serum stored for nine months. Virus of the Ind.C and NJ.M mouse strains was used and all sera were tested simultaneously in a single test with each strain.

Three pools of sera, each collected from 2 to 3 rabbits three weeks after an overt infection with the Ind.C strain, at a dilution of 1/5 neutralised 10,000 or more ID_{50} of the Ind.C strain and less than 100 ID_{50} of the NJ.M strain (no end-point). The same result was obtained with a pool of sera collected from six rabbits five weeks after an immunity test had indicated that they had developed a complete immunity against infection of the tongue epithelium from previous clinical or subclinical infections following inoculation with the Ind.C strain by various routes. These six rabbits were then infected on the tongue with the NJ.M strain and developed lesions. The pool of their sera collected three weeks later had the same neutralising activity against the Ind.C strain as before and also neutralised 100,000 or more ID_{50} of the NJ.M strain. The neutralising activity of the pooled sera of two of these rabbits collected 10 months later was the same. Both these rabbits developed lesions when inoculated intradermally at this time with the Ind.C strain and no change in the neutralising activity of their serum collected three weeks later was detected.

DISCUSSION

One of the reasons for an interest being taken in the characteristics of the virus of vesicular stomatitis is the importance of differentiating this disease in domestic animals from foot-and-mouth disease. A rapid diagnostic test which is now available for suitably

equipped laboratories is a complement fixation test using field samples of vesicular epithelium as antigen (Brooksby, 1948).

It is of interest to have demonstrated in the present studies a significant difference in the clinical syndrome produced in guinea-pigs after intradermal inoculation of the metatarsal pads with cattle strains of these two viruses. The well marked thermal reaction which accompanies the formation of the primary lesions only in the case of the virus of vesicular stomatitis is considered to be a characteristic which could be detected at the initial passage of field strains in guinea-pigs if they produced good local lesions. If standard immunological tests failed to identify a field strain as vesicular stomatitis or foot-and-mouth disease virus, as would occur if a new immunological type of either virus arose, the thermal reaction in guinea-pigs would be a useful rapid test to add to the various biological, physical and chemical tests which earlier workers had suggested as being of value. These tests have been listed by Brooksby (1948). In connection with the differential diagnosis of these two diseases the opportunity is taken to mention that in 1946 the writer made an intensive study of all the successful techniques which had then been used to demonstrate specific red cell haemagglutination by other viruses and applied these techniques to the viruses of vesicular stomatitis and foot-and-mouth disease without any success.

The appearance of well marked secondary tongue lesions in rabbits several days after the disappearance of small primary tongue lesions is of interest, since if a similar sequence of events were to occur in a naturally infected host it could lead to a false interpretation of the time of initial infection.

CONCLUSIONS

From a close study of the clinical syndrome in guinea-pigs following infection with the virus of vesicular stomatitis by the intradermal route three characteristics were noted which can be exploited to increase the value of this animal as an experimental host.

The marked rise in rectal temperature at the time of formation of a primary lesion was shown to be absent in the case of infection with the virus of foot-and-mouth disease and it is suggested that it could be used as a differentiating characteristic.

In guinea-pigs inoculated on both metatarsal pads with the same infective material the two primary lesions always erupted simultaneously or within a day of each other. Secondary lesions never appeared within two days of the eruption of the first primary lesion and in only 4 per cent of the guinea-pigs which developed secondary lesions did they appear within three days. It is possible, therefore, to count each inoculated metatarsal pad as a site of reaction and thus to obtain two observations from a single guinea-pig.

The low frequency with which secondary lesions occur in guinea-pigs, and the early production of an immunity following the infection of a single pad, enable a rapid cross immunity test to be carried out by confining the primary infection to a single pad and using the second pad for inoculation of the test virus.

It was confirmed that subclinical infections of guinea-pigs can arise from close contact with infected guinea-pigs. The route of infection in such cases could be intranasal as guinea-pigs were infected subclinically by this route with only ten or less intradermal infective doses.

Rabbits inoculated intra-epithelially on the tongue were as sensitive as guinea-pigs inoculated intradermally on the metatarsal pads for the detection of minimal infective doses of virus. The eruption of primary tongue lesions was accompanied by an inappetance, but only a slight rise in body temperature. The lesions healed very rapidly and were often followed in a few days by secondary tongue lesions the severity of which appeared to be inversely proportional to the severity of the primary lesion.

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THE *IN VITRO* SENSITIVITY TO NITROFURANS OF ORGANISMS ISOLATED FROM THE UTERI OF THOROUGHBRED MARES

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INTRODUCTION

Since Dodd and Stillman (1946) reported the antibacterial properties of certain nitrofurans, a number of compounds have been found valuable in both human medicine (Daily, 1948; Brennan, 1952) and in the treatment of animal diseases (Roberts, 1953; Williams Smith, 1955). Hasen and Moore (1954) and Kaplan and Hobgood (1954) described good results in the treatment of genito-urinary infections of man with one of these compounds (Furadantin). In thoroughbred mares uterine infections are sometimes encountered which fail to respond satisfactorily to surgical intervention or to various antibiotic and chemo-therapeutic measures. For this reason a study of the effects of nitrofurans on organisms recovered from the genital tracts of mares seemed worthwhile.

Dimock and Bruner (1949) and Cronin (1952) found streptococci (Lancefield's Group C) to be the organism most frequently isolated from the genital tracts of mares. While this species, therefore, has received the greatest attention in the present investigation, two other less frequently encountered organisms also were tested for sensitivity to certain nitrofurans. The pathogenicity of *Pseudomonas aeruginosa* has been recognized for a number of years (Dimock and Bruner, 1949) and Moran (1955) reported increasingly frequent isolations of fungi from the uteri of thoroughbred mares.

MATERIALS AND METHODS

In this area it is the practice for veterinarians to submit to the laboratory culture tubes inoculated directly from the genital tracts of mares according to the technique of Dimock and Edwards (1928). The organisms used in this study were obtained from these cultures. The media used were horse meat infusion agar (Dimock and Bruner, 1949) and, in cases suspected of fungal infection, Sabouraud's dextrose agar. After purification and identification, subcultures of bacterial strains selected for study were made in horse meat infusion broth and of fungi in Sabouraud's dextrose broth.

Seven nitrofurans were included in the investigation. These were Furacin, Furadantin, Furoxone, Furamazone, Furadroxyl, N.F. 291, and Furaspor. All drugs except Furadantin were dissolved in horse meat infusion broth and the solutions were then sterilized by Seitz filtration. Furadantin was first dissolved in polyethylene glycol 300 in a concentration of 0·5 per cent and final dilutions were then made in horse

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meat infusion broth and sterilized by filtration. Only freshly prepared solutions were used. Appropriate dilutions of the drugs were made in sterile infusion broth from the stock solutions and aliquots of these solutions were placed in sterile culture tubes (13 mm. x 100 mm.) so that the addition of culture inoculum brought the final volume of the inoculated material to 2·0 ml. in each tube, with the drug concentration at the required level.

The culture inoculum for each strain was prepared by diluting an 18-hour infusion broth culture with horse meat infusion broth to the required concentration. The tubes were inoculated with approximately 50,000 viable organisms. After incubation at 37°C. for 96 hours, examination for the presence of growth was made. The minimum bacteriostatic concentrations of the drugs were recorded as those in the tubes showing no visible growth. Subcultures were made from the tubes in which no visible growth appeared, and the minimum concentrations from which no organisms grew on subculture in infusion broth were recorded as the minimum bactericidal concentrations of the drugs.

In tests against fungi, final dilutions of Furaspor (the only nitrofuran tested against these organisms) were made in Sabouraud's broth and cultures were allowed to stand at room temperature for 48 hours before readings and necessary subcultures were made.

RESULTS

The concentrations of the drugs required to produce bacteriostatic and bactericidal effects are shown for each organism in Table 1. It will be noted that certain species were not tested with all drugs and that greater numbers of some species of organisms were tested with certain compounds. This arose because additional strains of organisms became available during the course of the work. The larger numbers of strains tested with some drugs include all strains tested with the other compounds.

Furacin and Furadantin showed fairly close similarity in their antibacterial activity, but Furadantin appeared somewhat more effective, particularly in its ability to produce bacteriostasis of beta-haemolytic streptococci in relatively low concentrations. Furthermore, although both compounds had a similar range of bactericidal concentrations, Furadantin showed a lower mean bactericidal concentration when tested against streptococci than did Furacin.

Furoxone was considerably more effective against streptococci than either Furacin or Furadantin. The results show that this compound, in the dilutions tested, was bactericidal rather than bacteriostatic, producing the former effect in low concentrations. N.F. 291 was the least active compound tested, and Furamazone was little more effective. Furadroxyl apparently was about equal to Furacin in its activity as judged by the results of these tests. As shown in Table 1, neither Furacin nor Furadantin showed any activity against *Pseudomonas* spp. in the highest concentrations tested. Furaspor was the only other nitrofuran tested with this species and, as shown in Table 1, it was moderately effective, producing bacteriostasis with the majority of strains in a concentration of 50 mg.

TABLE 1

IN VITRO TESTS OF NITROFURANS WITH MICRO-ORGANISMS FROM THE MARE GENITAL TRACT

Minimum inhibiting concentrations (mg. per 100 ml.) at 96 hours.

Nitrofuran	*Species*	*No. of strains*	*Bacteriostatic range*	*or Fungistatic mean*	*Bactericidal range*	*or Fungicidal mean*
Furacin	Beta-hemolytic streptococcus	28	0·5 — 20·0	3·0	0·6 — 20·0	4·1
Furadantin	„ „ „	28	0·4 —>20·0	> 2·4	0·4 —>20·0	> 3·0
Furamazone	„ „ „	15	— —	—	< 0·5 —>10·0	—
Furoxone	„ „ „	15	1·0 — 1·0	1·0	< 0·5 — 7·0	< 3·0
Furadroxyl	„ „ „	15	1·0 — 30·0	8·3	< 0·5 — 35·0	> 6·9
N.F. 291	„ „ „	15	5·0 — 12·0	8·3	6·0 —>20·0	>10·0
Furacin	*Pseudomonas aeruginosa*	5	>20·0 —>20·0	>20·0	>20·0 —>20·0	>20·0
Furadantin	„ „	5	>20·0 —>20·0	>20·0	>20·0 —>20·0	>20·0
Furaspor	„ „	9	40·0 — 70	50	50 — 100	75
Furaspor	*Candida parakrusei*	5	20 — 30	24	20 — 40	32
Furaspor	*Candida tropicalis*	10	20 — 20	20	20 — 30	27

>=greater than <=less than

per 100 ml. It was bactericidal to almost all strains at 80 mg. per 100 ml.

Furaspor also was tested against 10 strains of *Candida tropicalis* and 5 strains of *Candida parakrusei*. A fungistatic effect was evident at 20 mg. per 100 ml. and the drug was fungicidal at 30 mg. per 100 ml. except in the case of two strains of *C. parakrusei*, which were killed at a concentration of 40 mg. per ml.

The results obtained with Furaspor when tested with *Candida* spp. and *Pseudomonas* spp. seem sufficiently promising to warrant tests of this nitrofuran in mares harbouring these organisms in utero. While the results obtained with the streptococcal strains are not conclusive, they do reveal considerable in vitro sensitivity of certain strains, particularly to Furoxone and Furadantin. These two compounds also may prove valuable in the treatment of streptococcal endometritis in thoroughbred mares.

CONCLUSIONS

The results of in vitro tests of several nitrofuran compounds with bacteria and fungi isolated from the genital tracts of thoroughbred mares have been described.

Furaspor was effective *in vitro* against several strains of *Pseudomonas aeruginosa, Candida tropicalis,* and *C. parakrusei.* Furoxone and Furadantin exhibited good antibacterial powers in vitro against haemolytic streptococci (Lancefield's Group C).

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THE CHARACTERS AND DISTRIBUTION OF CERTAIN STAPHYLOCOCCI PATHOGENIC FOR THE BOVINE UDDER

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INTRODUCTION

Though much attention has been given to the value of individual tests as criteria for defining the pathogenic role of staphylococci, no satisfactory classification has emerged from the use of these tests. Strains of animal origin have been investigated by such tests as coagulase production, formation of toxin, pathogenicity for mice and fermentation reactions; and more recently, the sensitivity of these organisms to lysis by various bacteriophages has been investigated. The following account is given of previous work relevant to the present investigation.

The coagulase test has been accepted as an important criterion of pathogenicity and the ability or inability to coagulate plasma is regarded as a means of distinguishing pathogenic from non-pathogenic strains. But it is clear from the results of different workers that the reaction is dependent on the kind of plasma used in the test. Thus, Field and Williams Smith (1945) using the slide test found that of 20 bovine strains which coagulated human and rabbit plasma only 10 coagulated sheep plasma. Later, Williams Smith (1947) found that, of 41 bovine pathogenic strains coagulating human plasma within three hours in the tube test, 33 also clotted bovine plasma in 3 hours, but 8 needed 18 hours' incubation to produce clotting.

The production of both α and β haemolysins was shown by Minett (1937) to be a property of bovine strains. The characteristic appearance of colonies, however, was obtained on blood-agar only when the blood used contained no appreciable antitoxin. A further haemolysin designated δ (Williams and Harper, 1947) was found by Elek and Levy (1950) to be produced by 59 animal strains in addition to the other two haemolysins and appeared to be closely related to the pathogenicity of both human and animal strains.

The pathogenicity of staphylococci has been investigated by testing the lethal effects for laboratory animals either of whole culture or of the separated toxin. The results of Williams Smith (1947) showed that all of 30 cultures of pathogenic bovine strains prepared from agar slopes and injected intraperitoneally into mice were lethal within 48 hours. The centrifugates of 14 out of 20 broth cultures were toxic to mice in a dose of 0·4 ml. intravenously and the M.L.D. of a toxin was about 512 times its M.H.D.

It is generally held that fermentation reactions do not serve to dis-

tinguish pathogenic strains from non-pathogenic varieties. Fermentation of mannitol, as well as the property of gelatin liquefaction, is, however, recognised as a character of *Staph. aureus*.

Lysis by bacteriophage as a method of studying the epidemiology of staphylococcal infections in animals was first employed by Williams Smith (1948) who classified 1016 staphylococci isolated from bovine milk samples as follows:— 852 (83 per cent) were lysed principally by phage 42D and 96 (9·5 per cent) were lysed by phages of the group comprising 29, 29A, 31, 42C, 44, 52 and 52A. Subsequently, four broad groups of staphylococci have been recognised by phage typing of predominantly human strains (Rippon, 1956), of which Group IV comprises strains lysed principally by phage 42D. Groups I, II and III were often referred to in the past as the 52/52A, the 3A, and the 6/47 groups respectively and strains lysed by 42D were included in Group III. Edwards (1954) examined strains cultured from the milk of 37 cows undergoing therapeutic treatment with antibiotics. The strains from 35 cows, all belonging to one herd, were lysed principally by phage 42D and were thus of Group IV; with stronger phage filtrates, the phage pattern was typically 42D/54/77/79, i.e. there was lysis by phages of Groups I (79), III (54 and 77) and IV (42D). Of the remaining two cows, one yielded a staphylococcus lysed by phage 3A (Group II) and the other a staphylococcus lysed by 52B (Group III).

Thus it will be seen that staphylococci from the bovine udder have been examined by a number of cultural tests as well as by phage typing. The purpose of the present study was to examine strains isolated from the udder by both methods in an attempt to classify them.

METHODS

A total of 395 strains were obtained from the milk of cows mainly in three Compton herds, a few from cows in the isolation units at Compton, and the remainder from 19 herds on farms situated close to the Field Station. The majority were obtained from milk samples from cows at routine herd tests while a smaller number were recovered from abnormal secretion of cows affected with clinical mastitis. In the early part of the investigation strains were recovered from the milk of cows undergoing therapeutic treatment with antibiotics. In order to determine whether success or failure in treatment was correlated with the nature of the infecting strain, the tests employed were the coagulase test, phage type and sensitivity to antibiotics. Later, when it appeared that strains could be arranged into groups by the first two tests, further tests were employed on 64 strains, which included a representative number of strains belonging to each group.

Isolation. Strains were isolated by streaking one loopful (0·01 ml.) of gravity cream or abnormal secretion on the surface of the blood-agar medium recommended by Slanetz and Bartley (1953). This medium contained 2 per cent tryptose, 0·1 per cent glucose, 0·5 per cent sodium chloride and 2 per cent agar (pH 7·0); sheep or ox blood of low antitoxin content, i.e. not more than 0·25 units per ml. was added to 5 per cent. On this medium it was found that colonies of staphylococci developed the β type of haemolysis which is characteristic of most bovine strains on plates incubated for 36 hours at 37°C.

It was considered that for the purpose of identifying the phage type of the infecting strain, the isolation of one typical colony from quarter samples was sufficient, since Williams Smith (1948) and Price, Neave, Rippon and Williams (1954) have shown that infection is nearly always due to one type of staphylococcus. In herd tests where composite samples from the four quarters of each cow were plated, it was usual to examine at least two colonies from each sample. The presence of staphylococci outside the udder was detected by taking swabs from the site. Before application, the swab was moistened in sterile milk and then expressed into a small volume of milk containing 1:10,000 sodium azide. This sample was incubated at 37°C. for 6 hours, after which time one loopful was streaked on the surface of a blood-agar plate.

Coagulase production. The tube test using both rabbit and sheep plasma was carried out according to the method of Fisk (1940), except that the first reading was taken after 1½ hours incubation at 37°C. A number of strains were also tested for the yield of coagulase produced by an 18-hour culture. Serial doubling dilutions, using 0·4 ml. of culture, were made in saline and an equal volume of 1 to 5 plasma in saline was added to each dilution. The test was read after 1½ hours incubation at 37°C. and again after 3½ hours standing at room temperature when using rabbit plasma, and after 18 hours when using sheep plasma.

Phage type. Colonies of staphylococci which had been proved coagulase positive were checked for purity and then identified for phage type. The strains were tested first for sensitivity to phages employed at their routine test dilution and then in most cases to phages 1,000 times stronger than this.

Haemolysin production. The titre of soluble haemolysin was tested after incubating the strain for 5 days in Walbum's medium in an atmosphere containing 20 per cent CO_2. The supernatant was then tested in falling dilutions against rabbit and sheep erythrocytes using a technique similar to that described by Williams Smith (1947). The technique described by Elek and Levy (1950) was used for the study of haemolysin production on blood-agar containing a 5 per cent suspension of washed rabbit or sheep red cells; the basic medium employed however, was the same as that used for preliminary isolation. The surface of one half of each plate was spread with 7 to 9 drops of alpha antitoxin (1,100 units per ml.). After drying the plates at 37°C. for two hours, streaks of culture were made across the plate. The development of δ haemolysin was noted in the half containing antitoxin after incubating the plate for 48 hours at 37°C. in an atmosphere of 20 per cent CO_2.

Lethal toxin. Cultures grown in Walbum's medium for 5 days in 20 per cent CO_2 were passed through Seitz filter pads and the filtrate tested by injecting 0·2 ml. intravenously into each of five 20 g. mice. The titre of the toxin present in these filtrates was tested at the same time by its haemolytic effect on rabbit cells. The toxicity of the strain was judged by the deaths occurring during 48 hours in the inoculated mice.

Virulence of culture. Cultures were grown for 16 hours in Hartley broth and 0·2 ml., containing on average 100×10^6 organisms, was injected intraperitoneally into each of five 20 g. mice. Those strains found to be lethal within 5 days were retested by injecting cultures which had been freed from toxin by reconstituting the centrifuged sediment in fresh broth.

Fermentation reactions. The fermentable substances used were confined to glucose, salicin, arabinose, mannitol, inulin and raffinose. Readings were made after incubation for 5 days at 37°C.

Gelatin liquefaction. Two drops of an 18-hour broth culture were sown into 2 ml. of nutrient gelatin (20 per cent). The tubes were incubated at 37°C. for 5 days and maintained for 30 minutes at 4°C. before reading each day.

Sensitivity to antibiotics. One drop of an 18-hour broth culture was inoculated into a test medium consisting of Lemco broth containing falling concentrations of antibiotic. The inhibitory end-point was observed after incubation for 24 hours at 37°C.

RESULTS

Phage Patterns

From the results obtained on 381 strains, examined by the coagulase test and by phage typing (Table 1), it will be seen that five different phage patterns were distinguished among the strains, and for convenience of description these will be referred to in the paper as "types 1 to 5". There was considerable variation in the phage susceptibility of the individual strains and the phages listed in the table composing the "undiluted pattern" are those that formed the most typical patterns when tested with undiluted or "strong phages." Despite this variation, however, there was rarely any difficulty in assigning a staphylococcus to one of the five types.

Type 1. The first 68 strains examined in this investigation were isolated from 44 cows belonging to one Compton herd (herd A). They all belonged to the same phage type, being lysed by the phage 42D (Group IV); some were lysed also by strong phages belonging to Group I and III together and some by strong phages of Group III only. These strains coagulated rabbit and sheep plasma after 1½ hours incubation at 37°C.

The next 27 strains were isolated from 12 cows belonging to another Compton herd (herd B). While seven of these strains were identical with those of the first group, the other 20 strains differed by the fact that they failed to coagulate sheep plasma after 1½ hours incubation and only did so after standing for 18 hours at room temperature. When the phage sensitivity of the latter strains was examined it was found that though they were lysed by phage 42D, they were always lysed by certain phages which did not enter into the pattern of the first group; the most constant of these were phages 42E, 6 and 7. Because of these slight differences it was considered necessary to designate the 71 strains of this category as type 1a and the larger group of 231 strains as type I.

Type 2. Strains belonging to this type were isolated in the first place from the milk or secretion of cows belonging to another Compton herd (herd C). These strains were lysed by phages belonging to Group II typified by phage 3A. They coagulated

TABLE 1

PATHOGENIC STAPHYLOCOCCI PRESENT IN MILK

Classification of 381 strains by phage typing and the coagulase test.

Type	*No. of strains*	*Diluted*	*Phage pattern undiluted*	*Group*	*Coagulase production*					
					Tube test 1½ *hrs. at 37°C.*		*18 hrs. at R.T.*		*Titres*	
					Rabbit plasma	*Sheep plasma*	*Rabbit plasma*	*Sheep plasma*	*Rabbit plasma*	*Sheep plasma*
1	231	42D	29/52/79 42D/54/77	IV	+	+	+	+	500	8000
1a	71	42D	42D/6/7/42E/47	IV	+	—	+	+	250	200
2	46	—	3A/3B/3C/71	II	*—	—	+	—	100	0
3	19	52B	7/75/77/52B	III	+	—	+	—	1000	0
4	8	52A	52/52A	I	+	—	+	—	100	0
5	6	78	54/76/77/78	III	+	—	+	±	250	500

* + Human plasma after 1½ hrs. at 37°C. R.T. = room temperature.

rabbit plasma weakly after 18 hours at room temperature and produced coagulase in low titre. Fresh human plasma was more sensitive and was coagulated by these strains after 1½ hours' incubation. Strains of this type were also isolated from the hands of milkers.

Type 3. Strains of this type were isolated only from the milk of cows in herd C. They were lysed by phage 52B at routine test dilution and by other strong phages of Group III. These strains coagulated rabbit plasma but failed to react with sheep plasma; the titre on rabbit plasma was 1 to 1,000.

Type 4. Eight strains fell into this type. Their phage pattern was complicated but they could be assigned to one type because they were lysed by phages 52 or 52A (Group I) in dilute form. While coagulating rabbit plasma only in the tube test, their titre was low (1 to 100) and the clot produced was semi-solid.

Type 5. Six strains reacted with phage 78 (Group III). They failed to coagulate sheep plasma after 1½ hours incubation at 37°C. but did so after standing for a further 18 hours at room temperature, though the clot was semi-solid; they produced a moderate amount of coagulase in rabbit and sheep plasma.

A further 14 strains of coagulase-positive staphylococci isolated from normal milk were also submitted for phage typing. Five did not react with any of the regular phages employed; six, all from one herd, were lysed by phage 53 only and three were lysed by phages of Groups I and III and therefore resembled type I strains but did not coagulate sheep plasma.

Characteristics of Phage Types

A representative number of strains from each of the five types were examined by further tests to determine whether they possessed other characters that would distinguish them.

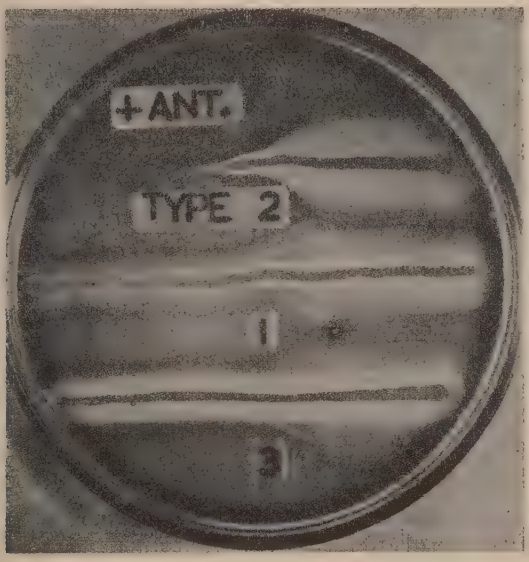
Haemolysin Production

Plate test. In common with the findings of Elek and Levy (1950) all the strains examined produced $\alpha\beta\delta$ haemolysins except those in type 2 which only produced α haemolysin. The amount of lysin produced, as judged by the diameter of the zone around the colony, varied with individual strains within types and, in particular, one strain of type 1 produced wide α haemolysis and little β haemolysis on sheep blood agar. Type 2 strains produced narrow α zones which, like those of other types, widened after plates had stood at room temperature.

Type 3 strains were distinguished by the broad zones of δ haemolysin produced while type 4 and 5 strains produced narrow zones. The difference in haemolysin production, particularly of the δ type, is shown in Fig. 1 where it will be seen that the type 3 strain produces a wide zone in the area of the plate containing α antitoxin.

Fig. 1.

Haemolytic character of staphylococcal types on blood-agar containing 5 per cent. washed sheep corpuscles.



The left side of the plate has been spread with α antitoxin, (1,100 units per ml.). The type 2 strain produces α haemolysin only, the type 1 αβ narrow δ and the type 3 αβ wide δ zone of haemolysis.

Tube test. Titres of α and β lysin produced in Walbum's medium varied considerably in strains of the same type and in different tests on the same strain. Strains of types 1 and 3 generally produced higher α haemolysin titres than the other types and one strain (S.67) of type 1 was notable in that it gave rise to a titre of 1 to 80,000. All strains with the exception of type 2 produced β haemolysin as shown by the "hot-cold" lysin test (Table 2).

Fermentation reactions. The limited number of test substances employed did not serve to distinguish the various types. All the strains examined were the same in that they fermented glucose and mannitol only and therefore conformed in this respect with the pathogenic species of staphylococci designated *Staph. aureus*.

Gelatin liquefaction. Only one type 1 strain (S.67) liquefied gelatin within 5 days incubation at 37°C. No strain in types 2 and 3 did so, whereas the eight strains in type 4 and six in type 5 liquefied this substance in 3 days.

Pathogenicity of Phage Types in Mice

Toxicity of filtrates. Table 3 shows that there is a close relationship between the α haemolysin titre of the broth filtrates of strains and their toxicity for mice. Thus, the results of all 64 strains showed that none of the 42 strains having titres below 1 to 1280 were toxic in a dose of 0·2 ml. intravenously whereas all 22 strains with titres of 1 to 2560 or over were lethal within 24 hours of the time of injection. These findings agree with those of Williams Smith (1947) and show

TABLE 2

HAEMOLYSIN PRODUCTION AND GELATIN LIQUEFACTION

Phage type	*No. strains tested*	*Haemolysin production*			*Gelatin liquefaction*
		Plate	*Tube (average titre)* *Rabbit cells* (α)	*Sheep cells* (β)	
1	15	αβδ	640-5,120	40-320	— (1+)
1a	15	αβδ	640-5,120	40-320	—
2	10	α	160-2,560	0	—
3	12	αβδ	640-5,120	20-80	—
4	8	αβδ	160-640	20-80	+
5	4	αβδ	320-640	20-80	+

TABLE 3

RELATIONSHIP BETWEEN α HAEMOLYSIN TITRE, TOXICITY AND VIRULENCE FOR MICE OF PHAGE TYPES

Phage type	*No. tested*	*No. strains of each type which were toxic when 0·2 ml. of filtrate was injected intravenously.*					*No. of strains lethal to mice in a dose of 0·2ml. culture intraperitoneally*
		α-haemolysin titre 0-320	640-1280	2560-5120	5120	*Total toxic*	
1	15	0/1	0/7	6/6	1/1	7	1
1a	15	0/2	0/3	8/8	2/2	10	0
2	10	0/7	0/2	1/1	—	1	0
3	12	—	0/8	4/4	—	4	6
4	8	0/8	—	—	—	0	8
5	4	0/4	—	—	—	0	4
Total	64	0/22	0/20	19/19	3/3	22 (34·4%)	19 (30%)

that the M.L.D. of a filtrate is approximately 512 times its M.H.D. A few strains were observed to be toxic in a dose less than 0·2 ml. and the M.L.D. of S.67 (type I) was found to be 0·05 ml. Only about one third of all the strains tested were toxic and there was a tendency for strains of type I to be more toxic than strains of the other types.

Virulence of culture. It will be seen that all the type 4 and 5 strains were pathogenic and only one of type 1. Type 3 strains varied in this respect and of the 12 strains tested 6 were pathogenic. Type 4 and 5 strains killed mice within 12 hours, whereas those of type 3 were not lethal so quickly. The lethal effect of strains was not influenced by the small concentration of toxin present in the broth culture after 16 hours incubation, since suspensions of washed organisms produced the same result as whole culture. It will be noted that only 19 (30 per cent) of the 64 strains were pathogenic whereas Williams Smith (1947) found all of 30 strains pathogenic when using a dose approximately eight times as large as that used in this test.

There was no correlation between toxicity and virulence for mice of the phage types in the doses employed. Type 1 strains were relatively toxic but non-virulent whereas types 4 and 5 were non-toxic and yet highly virulent. By referring to Table 2 it will be found that there is a relationship between virulence and power to liquefy gelatin in strains belonging to types 4 and 5.

Antibiotic sensitivity. Of the 120 strains which were tested for sensitivity to penicillin only 14 proved resistant to concentrations as high as 10 to 200 μg./ml. Moreover, it will be seen in Table 4 that all the resistant strains belonged to type 3. These strains also produced penicillinase and all were isolated from the milk of cows

TABLE 4

SENSITIVITY OF STAPHYLOCOCCI TO ANTIBIOTICS

Type	*Penicillin*			*Aureomycin*		
	Strains tested	*Sensitive 0·125 μg./ml.*	*Resistant 10-200 μg./ml.*	*Strains tested*	*Sensitive 1·25 μg./ml.*	*Resistant >1·25 μg./ml.*
1	48	48	0	21	21	0
1a	32	32	0	—	—	—
2	18	18	0	1	1	0
3	14	0	14	11	11	0
4	4	4	0	—	—	—
5	4	4	0	—	—	—
Total	120	106	14	33	33	0

belonging to herd C. Only 33 strains were tested against aureomycin including eleven type 3 strains, and none was found to be resistant.

Distribution of Phage Types in the Milk of Cows and in other Sites

Milk

By referring to Table 5 it will be seen that some strains of all the types were associated with clinical disease and that out of a total of 266 cows infected with staphylococci 50 (19 per cent) were showing clinical mastitis. Strains of the first three types were isolated from subacute clinical cases usually occurring at the time of calving, but two strains included in type 4 and three in type 5 were responsible for an acute form of disease accompanied by constitutional symptoms. Strains belonging to type 1 comprised 302 (77 per cent) of the total strains isolated, and the fact that they were the predominant type agrees with the results of Williams Smith (1948) who examined the phage type of a larger number of strains.

TABLE 5

ORIGIN AND ASSOCIATION OF VARIOUS PHAGE TYPES WITH CLINICAL MASTITIS

Phage type	*Origin*	*No. strains examined*	*No. of cows from which strains were isolated*	*No. of cows showing clinical mastitis*
1	Herd A	159	100	14
	Herd B	36	27	5
	Herd C	11	11	3
	Other Herds (12) ..	25	25	0
1a	Herd B	62	39	5
	Other Herds (5) ..	9	9	4
2	Herd C	46	30	6
3	Herd C	19	11	6
4	Other Herds (3) ..	8	8	4
5	Isolation Units ..	6	6	3
	Total	381	266	50

Herds A, B and C were examined at six-monthly intervals for the presence of staphylococcal infection in composite milk samples taken from the cows. The herds were free from infection with *Str. agalactiae* and no special precautions were practised to prevent the spread of staphylococcal infection. The results of successive herd tests are shown in Table 6. It will be seen that these three herds were heavily infected with staphylococci belonging to three of the types described. In Herd A only type 1 strains were found; infections in cows in Herd B were due either to type 1 or type 1a and occasionally,

individual cows were found to be infected with both these types. In Herd C, infection with three of the phage types was present; though a few of the cows were infected with type 3 penicillin resistant staphylococci, infection in the main was due to type 2 staphylococci. In the last two tests cows harbouring type 1 infection were found while a few cows with mixed infections have also been encountered.

TABLE 6

PREVALENCE OF STAPHYLOCOCCAL PHAGE TYPES IN THE MILK OF COWS

Herd	*Test*	*Cows tested*	*No. positive staphylococci*	*Phage type present in milk*				
				1	*1a*	*2*	*3*	*mixed types*
A	1	84	41	41	—	—	—	—
	2	59	30	30	—	—	—	—
	3	87	50	50	—	—	—	—
	4	78	34	34	—	—	—	—
B	1	50	28	14	11	—	—	3
	2	65	20	5	15	—	—	—
	3	76	55	16	38	—	—	1
	4	70	60	10	49	—	—	1
C	1	35	12	—	—	8	4	—
	2	37	25	—	—	20	4	1
	3	39	21	2	—	17	2	—
	4	46	38	6	—	26	—	6

Other Sites

Studies on the distribution of *Str. agalactiae* (Rpt. 1944; Chodkowski, 1949) have shown that this organism is found in sites outside the udder, notably on the teat surface, on the hands of the milkers and on the surface of utensils. With regard to staphylococci, McDiarmid (1947) found these organisms present on the teats of cows; Spencer and Lasmansis (1952) found the principal reservoirs to be the skin of the teats and cups of the milking machine while the hands of the milkers employed in the herd were not infected. A search was made for the presence of pathogenic staphylococci on the surface of the teat and on milkers' hands.

Teat. Composite milk samples from the four quarters of each of 20 cows in Herd A were made daily on six consecutive days. At the same time swabs were taken from the skin of the four teats and examined by the technique described earlier. The results obtained are shown in Table 7. In 14 of the cows type 1 staphylococci were found in the milk at every test in 9 cows and in three or more tests in the remainder, while no infection was found in 6 cows. It will be seen that infection with type 1 staphylococci was nearly always present on the surface of the teats of the cows whose milk was infected and in half of the 36 swabs taken from the 6 cows whose milk was free from infection. McDiarmid (1947) also found that the presence of staphylococci on the teat was not always accompanied

by infection of the udder and that the carriage of pathogenic staphylococci on the teat was intermittent.

TABLE 7

PRESENCE OF TYPE CI STAPHYLOCOCCI IN THE MILK AND ON THE TEAT SURFACE

Cows examined	*Milk samples infected*	*Teat swabs infected*
14 infected cows	72	65
6 non-infected cows	0	18

The presence of pathogenic staphylococci so frequently on the teats of cows whose milk was not infected might be explained by their recent implantation by the infected liners of the machine during milking. This was demonstrated by examining 11 milking machine clusters during use in Herd A. From 65 swabs taken from these clusters on 6 separate daily examinations, staphylococci were found in 38 swabs.

The persistence of staphylococci after implantation on the teat surface was determined by contaminating the teats of 10 dry cows with a culture of a type 1 strain suspended in milk. By examining swabs daily from the teat surface it was found that the organisms persisted for an average period of six days with a maximum period of nine days. This represents a survival time which is slightly longer than the 3 to 4 day period given by Chodkowski (1949) for *Str. agalactiae.*

Milker's hands. Swabs were taken on separate days from the hands of milkers engaged in milking in the three herds (A, B and C) and examined for the presence of the phage types of staphylococci found in the milk of the cows. The results of these tests are shown in Table 8. It will be seen that there was a high incidence of contamination on the hands of the milkers engaged in Herds B and C and it was found that the staphylococci present were of the same types as those found in the milk of the cows. A lower incidence was

TABLE 8

INCIDENCE OF STAPHYLOCOCCAL TYPES ON MILKERS' HANDS

Herd	*No. milkers*	*No. swabs*	*Total swabs positive*	*Staph. types present in swabs*			
				1	*1a*	*2*	*3*
A	7	41	11 (27%)	3	—	8	—
B	6	61	48 (79%)	15	33	—	—
C	5	28	24 (86%)	6	—	18	—

found on the hands of milkers engaged in Herd A and here type 2 staphylococci were usually present although they had not been detected in the milk of the cows.

DISCUSSION

By the application of phage typing it has been possible to distinguish five main types of pathogenic staphylococci which can be isolated from the bovine udder and the distinction made by this method can be confirmed by the possession of other characteristics in the types. It is realised, however, that other varieties may exist since this investigation has been limited principally to a close study of the staphylococci in three herds supplemented by the examination of representative strains from nineteen other herds. But, by a limited study of this kind it has been possible to define at least some of the staphylococci which are in fact pathogenic or potentially pathogenic for the udder, since strains from all the types were found in cases of clinical mastitis or in chronic affections of the udder.

In studies on staphylococci in general, it has been customary to define organisms of this species as pathogenic or not according to their reaction to certain laboratory tests rather than by the criterion of host pathogenicity. For instance, it has been widely accepted that coagulase production is a characteristic of pathogenic strains but no standard for the application of the test or even of the plasma to be used has been laid down. As a result, previous workers have noted the different effects produced by staphylococci from various animal sources on different plasmas and after various times of contact but have not related these differences to different varieties. The fact that the sensitivity of rabbit and of sheep plasma varies has been found a useful distinguishing test and, in combination with phage typing, has formed a basis for the classification of the strains examined. All five types could be isolated from cases of clinical mastitis and could also be defined as pathogenic by the coagulase test, except type 2 staphylococci which were very weak coagulase producers. Though this test therefore proved of value for confirming the pathogenicity of strains, it may well be that a larger survey might have revealed strains pathogenic for the udder which did not have the power to coagulate plasma.

Lack and Wailling (1954) have also criticised the acceptance of laboratory tests as criteria of pathogenicity and in particular have stated that α haemolysin production is not always a property of human pathogenic strains, since they noted its absence in 18·5 per cent of strains causing osteomyelitis. This finding was confirmed in this study since strains falling into types 4 and 5 which were low α haemolysin producers and non-toxic to mice could be found in acute cases of mastitis, whereas strains belonging to type 1, which were high α haemolysin producers and toxic, were not usually associated

with acute clinical disease. On the other hand, the severity of the disease was associated with a marked virulence of the culture for mice and strains belonging to types 4 and 5 which were lethal in this way produced moderate amounts of δ haemolysin and rapid liquefaction of gelatin.

The epidemiology of staphylococcal infection in three herds confirmed the findings of Williams Smith (1948) and of Price *et al.* (1954), who showed that there was a predominating type in individual herds which varied in different herds and that more than one type might be present. In two of the herds in the present study the prevailing infection was due to a staphylococcus characteristically lysed by phage 42D, referred to for convenience as type 1, while in the third herd the predominant infection was with staphylococci lysed by strong filtrates of phages 3A/3B/3C and 71 (type 2) with a low incidence of infection by type 1 and with staphylococci lysed characteristically by phage 52B (type 3).

In a search for pathogenic staphylococci in situations exterior to the udder, it was found that they could be isolated with regularity from the skin of the teat, from the liners of the milking machine and from the milkers' hands, thus showing that they have the same distribution as *Str. agalactiae.*

CONCLUSIONS

A total of 395 strains of staphylococci were isolated from the milk or abnormal secretion from cows in three Compton herds and in isolation units at Compton, and from cows in 19 other herds.

By phage typing methods it was possible to divide 381 of these strains into five main types, while the remainder either did not fall into these types or were not typed by the phages employed. Of the typed strains 302 (77 per cent) were lysed predominantly by phage 42D (type 1 and sub-type 1a); 46 (12 per cent) by strong filtrates of phages 3A, 3B, 3C, and 71 (type 2); 19 (5 per cent) by phage 52B (type 3); and the remaining 14 strains (3·5 per cent) by phages 52A (type 4) or 78 (type 5). In recognising the five types consideration was generally given also to the patterns of lysis with the strong filtrates; considerable variation in these patterns was observed.

Staphylococci of these types gave constant reactions to the coagulase test with both rabbit and sheep plasma and this test formed a useful method of confirming the identity of phage types.

A relationship was found between the α haemolysin titre and the toxicity of strains for mice but there was no correlation between the toxicity and the virulence for mice of the different phage types when doses of 0·2 ml. toxin and of culture were employed. There was a tendency for the virulence for mice of types 4 and 5 to be related to the severity of the mastitis caused by these strains and with the marked production of δ haemolysin and the power to liquefy gelatin.

It was found that the cows in two of the herds examined were

infected only with staphylococci of one type (type 1) while those in a third herd were infected with three types (types 1, 2 and 3).

Staphylococci commonly pathogenic for the udder were found regularly on the skin of the teat of infected cows and frequently on the teats of those not infected; they were also found on the hands of the milkers and on the liners of the milking machine.

Out of 120 strains examined, 14 strains, all belonging to type 3, were resistant to penicillin; 11 of the 14 strains were tested against aureomycin, but none were resistant.

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THE BLOOD GROUPS OF THE PIG

V. Furthur Observations on the Epidemiology of Haemolytic Disease in the New-born

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INTRODUCTION

Goodwin, Saison and Coombs (1955) observed that red cell iso-antibodies were very common among pigs that had been vaccinated twice or more with crystal violet swine fever vaccine. Later work has indicated, however, that there is a much lower incidence of iso-antibodies among Large White than among Essex or Wessex sows when the red cells used for testing are from Large White boars (Goodwin and Saison, 1956). As the Large White x Large White and Essex/Wessex x Large White matings probably account for the greater part of all the pig matings in this country, and whereas the original survey (Goodwin *et al.*, 1955) was almost confined to the sera of Essex/Wessex pigs, it follows that the over-all incidence of iso-antibodies reactive against the cells of breeding boars is not as high as was first believed. Nevertheless, the number of sows with high iso-antibody titres is still much greater than the number of litters that has been reported as suffering from haemolytic disease and this disparity has been the stimulus for a wider enquiry into the epidemiology of the condition.

In the work now described we sought to determine whether the incidence of haemolytic disease was indeed low and, at the same time, to observe some of the factors that governed the risk to a litter from a vaccinated sow. With regard to this second aim, it was recognized that the lack of knowledge of the individual red cell antigens might prove a handicap but the questions already posed could not wait upon the results of a long-term study of pig blood groups. Furthermore, in the routine field use of crystal violet vaccine on many thousands of pigs there arose concurrently an experiment in iso-immunization the scale of which could not be repeated solely as a research problem and, as an alternative lapinized vaccine was being developed, there was added reason for not delaying this epidemiological study.

Although none of the work described in this series of papers precludes the existence of a naturally occurring form of haemolytic disease, it has been assumed from the fact that all the cases known to us have occurred in litters from vaccinated sows (or sows in which previous vaccination cannot be discounted) while iso-antibodies have been found to be both rare and at very low titres in unvaccinated pigs, that for all practical purposes haemolytic disease in the new-born pig can be regarded as an artificially induced disease. Should a condition arise that was undisputedly a spontaneous form of the disease it would need to be studied as a separate entity, for the conclusions reached here apply only to the condition that is the result of vaccination.

It is necessary to define the term 'haemolytic disease', because the

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description is often confined to those new-born pigs that die as a result of the haemolytic process. It has been assumed that the existence of iso-antibody in the circulation of the new-born is pathological and, therefore, any animal that has demonstrable circulating iso-antibody is judged to be suffering from haemolytic disease. It follows that there will be all gradations of severity of the disease between animals that are otherwise apparently normal and those that die.

Most of the present results were obtained with samples from Essex or Wessex sows and they refer, therefore, to that section of the pig population which appears to be most at risk.

MATERIALS AND METHODS

Pigs. The animals used have been so varied that they cannot be described collectively. Instead, mention is made later in each section of the results of the pigs from which samples were obtained and the reasons for their selection.

Biological samples. Whole blood, serum and red cell samples, as also the milk samples, were obtained in the manner already described (Goodwin *et al.*, 1955; Goodwin and Coombs, 1956).

Serological techniques. The techniques used for the preparation of samples and the performance of the direct agglutination (D.A.), indirect anti-globulin sensitization (I.S.T.) and A/O grouping tests have been described elsewhere (Saison, Goodwin and Coombs, 1955; Goodwin *et al.*, 1955). The direct anti-globulin sensitization test was performed after washing the red cells four times in 0·9 per cent saline to remove all traces of serum, and making them up approximately to a 10 per cent suspension. One drop of this suspension was added to one drop of a rabbit anti-pig globulin serum on a tile. The results were classified from strong to weak according to the size of the agglutinates, or as negative when the suspension remained unchanged after ten minutes.

During this work, however, a serious problem arose in the laboratory tests. The selection of a standard multiple-antigen group O cell has been described in an earlier paper (Goodwin *et al.*, 1955) and this cell had been used extensively for testing. It was intended that it would continue to be a reference cell and thus provide one constant factor in an uncharted antigen-antibody system. In due course, however, the cell became unsatisfactory, in that after washing and before exposure to a pig serum it reacted in the presence of anti-globulin serum as if it were weakly sensitized. Cells from other pigs that had been bled repeatedly likewise deteriorated, although their deterioration occurred later. (None of our pigs has been bled so frequently or for so long as pig 57 which supplied the standard cell.) Various modifications were made in the testing reagents and the bleeding technique but all these attempts to overcome the difficulty were unsuccessful. It was noteworthy that throughout the period when the several different reference cells were giving unsatisfactory results the tests using cells from local boars in the field were nearly always satisfactory, despite the fact that these samples were generally obtained in more hurried and less clean circumstances than on our own farm. Some of this work could not be continued until new reference cells were used and this necessitated cross-checking titra-

tions of certain sera against both the old and new cells as described later. None of the unreliable titrations has been included in the results.

Haemoglobin estimation. The concentration of haemoglobin in the tail blood of piglets was estimated colorimetrically at 540 mμ after taking 20 cu. mm. of blood into 4 ml. of 0·4 per cent ammonia.

RESULTS

Maintenance of Iso-antibody Titre

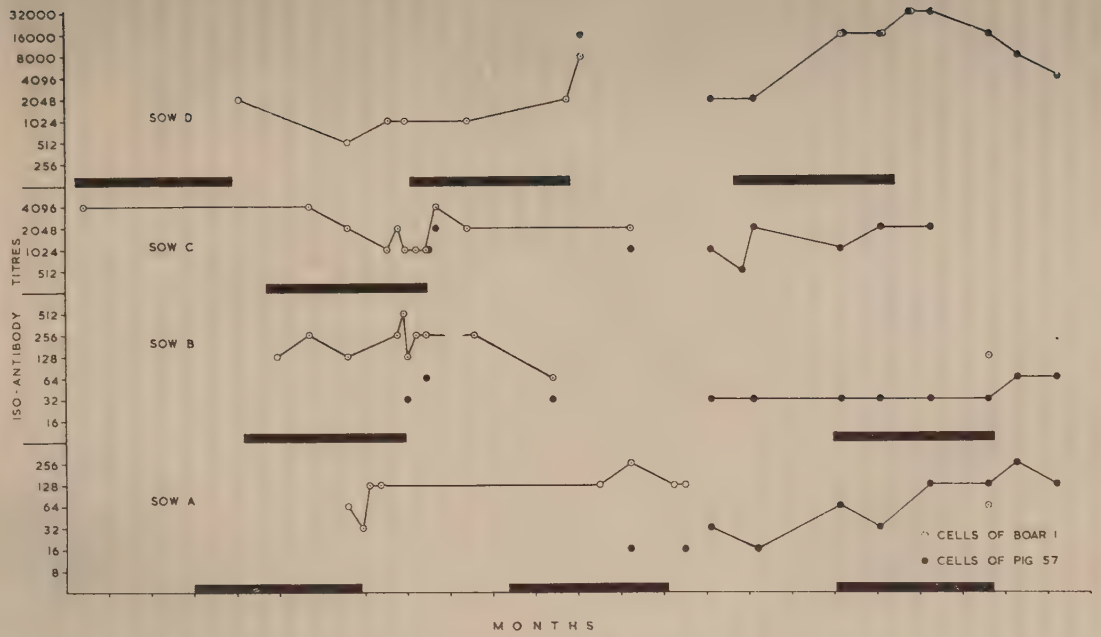
Early in this work it had been observed that haemolytic disease could be readily reproduced in successive litters from the same mating, and this had been attributed to transplacental stimulation. When the antigenic properties of crystal violet vaccine became known, however, the fact that the sows in our possession continued to afflict their litters with the disease, even though they had not been re-vaccinated for one to two years, directed attention to the possibility that serum iso-antibodies might persist at high titre for many months.

To study this point, four Wessex sows whose litters had been clinically affected were bled frequently over a period of about eighteen months (Fig. 1). Although both direct agglutination and indirect sensitization tests were performed, the results of only the latter titrations are given. All the matings were to a Large White boar whose cells were used for the initial titrations. Unfortunately, weekly bleedings caused this boar to become savage and thereafter the standard cells from pig 57 (Goodwin *et al.*, 1955) had to be used instead. Some of the early serum samples were re-titrated using the latter cells and, as an opportunity arose later to re-bleed the boar on a single occasion, a cross-check was made by titrating some of the later sera against the original boar's cells (Fig. 1). All four sows were of group A.

Sow A showed a stable titre to the boar's cells of about 128 for eight months and a sample tested at fifteen months gave a titre of 64. The values obtained with the cells from pig 57 showed an increase during the last pregnancy* but as the values obtained with the two testing cells did not at first agree, the significance of this rise is questionable. The titre of sow B was 128 to 256 during the first pregnancy and the same seventeen months later. During this time there was an interval of ten months between pregnancies. With sow C the titrations made with the cells of the boar and pig 57 gave almost identical results. Despite the fact that this animal was unmated for the last twelve months, the titre remained fairly steady at about 2048 throughout the period of observation (twenty months). In sow D, where the titres recorded by the two testing cells were also very similar, the antibody titre did not decline over a period of nineteen months, being 2048 when first examined and

*The terms 'last pregnancy', 'first pregnancy' etc. refer to the pregnancies shown in Fig. 1 and not to the complete breeding life of these sows.

Fig. 1.



Maintenance of iso-antibody titre in four Wessex sows; A, B, C and D.
Note: The gestation periods are shown as black blocks.

4096 in the final sample. In this animal there was an interesting variation that was not seen in sow C but may have been present in a very mild degree in sows A and B. The titre fell slightly after the first pregnancy but increased steeply at the end of the second. It fell again between the second and third pregnancies, only to rise even more markedly during the latter pregnancy reaching a peak of 32,000 in the sample taken closest to parturition. This peak titre was maintained for about a month and then fell away again.

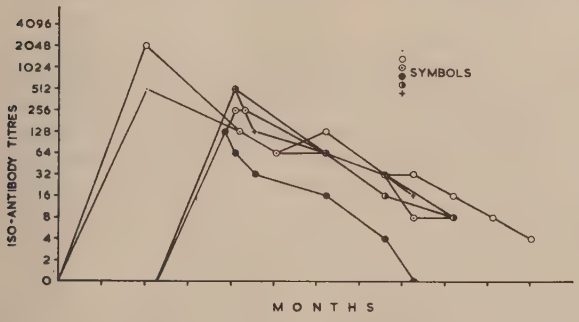
The main conclusion from the above observations was that in sows whose litters showed varying degrees of haemolytic disease the iso-antibody titre could be maintained for long periods even in the non-pregnant animal. Sow C was said to have been vaccinated last in February 1952 and the antibody titre was not declining two and a half years later. Likewise, the last recorded vaccination of sow D was in January 1952 and as this animal had a litter affected with haemolytic disease in May 1955, it is probable that a high iso-antibody titre has been maintained for three and a half years or more. The behaviour of the serum antibody level in sow D, although suggestive of antigenic stimulation during pregnancy could also be a non-specific effect associated with the approach of parturition and it is difficult to evaluate these variations in a single animal.

In sharp contrast to the results given by sows A, B, C and D were the findings after experimental vaccination (Fig. 2). Again only the indirect sensitization test titrations are shown. These animals were all the offspring of Essex x Large White matings and they were first subjected to an intensive course of vaccination (four or six injections of vaccine at fortnightly intervals) when about six months old. In all six animals the iso-antibody titre was not maintained; instead, it declined steadily and uniformly. It seemed possible that this type of antibody response might arise after a single vaccination and that the initial course of injections were more comparable with a single injection than with the injections given annually in the field. Consequently, four of the six pigs were re-vaccinated about six months after the first injections and the iso-antibody response was again followed. Now, however, the cells of pig 57, which were used satisfactorily for the titrations shown previously (Fig. 1) were deteriorating and other cells had to be used. Cross-checking titrations were made but to illustrate these would complicate the graph unnecessarily. The general conclusions on the second iso-antibody response can be summarized as follows. All four animals responded with a rapid increase in iso-antibody titre, from the low pre-vaccination titres of about 8 to 19 to peak post-vaccination titres that were close to the peak titres shown in Fig. 2. Thereafter, the titres declined as rapidly as after the first response.

Thus the iso-antibody response in experimentally vaccinated pigs proved to be different from that seen in sows vaccinated in the field whose litters had been affected with haemolytic disease, and

the explanation for this observation was sought in other differences between the two groups of animals. Fortunately, however, this difficulty was soon resolved by a further sow that had recently lost a litter with haemolytic disease. This animal, also a pure Wessex, had been vaccinated once and had then reared all her first litter. Four months after her second vaccination her second litter was lost with haemolytic disease. A week later the serum iso-antibody titre was 8000 (I.S.T.). She was re-mated to another boar and the titre was followed during gestation, using the cells of the sire for testing. The titre declined throughout pregnancy in a manner similar to that shown by the pigs experimentally vaccinated (Fig. 2) and shortly before term it was only 16. The sow died just before farrowing was due, but there is no doubt that the litter would have been almost unaffected by the existing antibody level.

Fig. 2.



Decline of iso-antibody titre in six bacon pigs vaccinated experimentally. Note: Some sections of the graphs coincide.

Considering the above observations as a whole, the simplest explanation appeared to be that after vaccination the iso-antibody titre soon reached a peak but, thereafter, it either declined rapidly or remained high. As those sows in the field that maintained their antibody titre would have a greater chance of affecting their piglets or becoming conspicuous by repeatedly losing their litters, it would be understandable if most of the animals in our possession with a history of haemolytic disease were of this type. Indeed, additional evidence has since been obtained which supports this contention. Several more sows have been seen showing each type of antibody response. Initially they were indistinguishable, all having lost piglets following vaccination, but whereas some continued to lose their litters, the others showed low antibody titres by the next farrowing and, in the absence of further vaccination, they have reared their piglets normally. Although the latter type of sow has been less commonly brought to our notice, it does not follow that

this type of antibody response is unusual. So far, no evidence is available to suggest that these two types of response depend on factors other than the sow itself (such as the types of antibody); both types of response have been seen in Essex/Wessex pigs and also within a single herd. Further observations on the persistence of the iso-antibody titre may be found in the following section.

Incidence of Iso-antibodies and Haemolytic Disease in Selected Herds

Some findings are now given for five herds in which observations were made on the distribution of iso-antibody titres and the relationship between these titres and the incidence of haemolytic disease. The first two herds (A and B) had no history of the disease and they were, therefore, of special interest. In view of the many visits that had to be made, the herds were selected mainly for their convenience; even so, various practical difficulties beyond our control prevented us from making all the observations that we had wished. All the titres quoted were obtained with the indirect sensitization test.

Herd A. This was a small purebred Essex herd of eight sows and a boar. Four of the sows had high serum titres (512) against the boar's cells, the samples being taken twenty weeks after the last vaccination in three cases and eight weeks after in the fourth. The first sow farrowed five weeks after sampling and the litter was examined in detail. During the first twenty-four hours, when mild signs of haemolytic disease appeared, three of the thirteen piglets were bled and all had very strongly sensitized cells. During the second day of life several piglets showed obvious clinical signs, mainly of jaundice, but in one animal there was marked pallor associated with a haemoglobin level of 3·6 g./100 ml: thereafter recovery was rapid. None of the piglets appeared to be inconvenienced by the disease and all recovered. This observation established that a sow selected as a result of a high serum titre could have a litter obviously affected with haemolytic disease. Yet the signs were not at first apparent to the owner and had the litter not been the subject of special examination it would have been left undisturbed and reared without knowledge of the disease.

It was impossible to examine the litter of the second sow but the owner reported that two of the piglets were obviously jaundiced and one died; the serum titre was 1024. The third sow farrowed with a serum titre of 256; the litter was not clinically affected and only some of the piglets had sensitized cells. There was evidence that several pigs in this litter had not suckled normally. The fourth animal had an apparently normal litter but the serum titre had declined to 64 at farrowing.

Herd B. This herd of about twenty breeding stock was predominately of the Essex breed and two boars (Large White and Essex) were in use. Vaccination was at approximately six-monthly intervals and consequently more intensive than usual. The whole herd was examined for iso-antibody titres, using the cells of both boars for testing, and most of the results are shown in Table 1.

It can be seen that antibodies were common and often at high titre but there was a marked difference in the titres recognized by the two boars, the cells of the Essex boar detecting much less antibody than those

TABLE 1

ISO-ANTIBODY TITRES IN HERD B

Sow	*Interval since last vaccinated (weeks)*	*Iso-antibody titre*		*Titre 6-8 months later (before next vaccination)*		*Titre two weeks after this vaccination*	
		against Large White boar	*against Essex boar*	*against Large White boar*	*against Essex boar*	*against Large White boar*	*against Essex boar*
281	4	32	16	4	8	<32	16
444	6	32	0	32	0	64	1
278	13	512	64	128	<8	1024	4
489	14	8	0	8	1	8	1
621	13	4	0	8	0	256	0
652	14	64	0	128	1	256	1
653	14	128	128	64	32	512	128
AA5	13	256	32	32	16	128	32
314	4	>512	256	128	64	8000	512
A1	13	>512	0	>512	0	4096	0
100	13	512	>256	64	<32	512	256
B8	6	256	128	256	64	1024	512
C1	14	<2	0	1	1	64	1
C5	4	128	8	16	8	512	64
101	13	16	128	8	8	256	64
873	4	2	0	0	1	0	4
148	4	>256	0	64	0	64	1
102	4	32	0	Not re-vaccinated			
104	4	16	0				
105	4	128	1				

of the Large White. One animal (sow 873) was conspicuous by its lack of antibodies and this animal is the only Large White sow in the table. Seven of the seventeen sows that were examined later showed a clear decline in titre (against the cells of the Large White boar) between the two vaccination dates. In general, the response after the last vaccination was similar to the earlier pattern and this was so for the titres against both boars. The improved titres after the last vaccination in some cases compared with the original values is probably due in the main to the shorter interval between vaccination and sampling on the second occasion.

The concurrent use of the Essex boar in this herd reduced the over-all risk at farrowing but there were at this time six animals whose serum titre was 128 or more against the cells of the boar that had served them: sows 278 and A1 (both in-pig to the Large White boar) and sows 653, 314, 100 and B8 (all in-pig to the Essex).

Sows 653 and B8 farrowed one month and nearly six months, respectively, after the first samples were obtained and the serum titre of both was 32. The litters were clinically normal and the haemoglobin concentrations were high; in the first litter some of the piglets had weakly sensitized cells on the second day, but in the second litter there was no such sensitization after about twelve hours of suckling.

Sow A1 farrowed exactly five months after the first serum sample was obtained. The titre at farrowing was 512 to 1024. Ten piglets were bled at twelve to twenty-four hours and the cells proved to be strongly sensitized in every case. The litter was bled a further twenty-four hours later when the mean haemoglobin concentration was 9·5 g./100 ml. Clinically, the pigs although robust were not absolutely normal and for three days a slight yellowness was present in the skin, which coupled with the pinkness due to the high haemoglobin concentration gave the litter a peach colour.

Sows 278 and 100 farrowed within twenty-four hours of each other just six months after being first bled. Use was made of this to compare the two litters, as the serum titres at farrowing were 256 and 32, respectively. Although the litter from sow 278 all showed strongly sensitized cells on the second day of life (less strongly sensitized however, than the cells from the litter of A1), while the litter from sow 100 was completely negative when similarly tested on the second day, the haemoglobin concentration in litter 278 fell, on the average, by only 0·9 g. between the second and sixth day of life. Furthermore, there was virtually no difference between the mean haemoglobin concentration of each litter when sampled after five days (9·1 and 9·3 g./100 ml., respectively).

Detailed observations were not made on the litter from sow 314 but the piglets appeared normal clinically. Most of the other litters farrowed in this herd during the same period were examined clinically and no piglet losses occurred that could be associated with haemolytic disease.

In summary, these farrowings had demonstrated several points. First, haemolytic disease as judged by the presence of sensitized neonatal cells was occurring in the herd. Secondly, the only sign of this disease was very slight jaundice in a few piglets; this would almost certainly have passed unnoticed by observers unaccustomed to the disease in its milder forms. Thirdly, the mildness of the disease was aided by the not uncommon tendency of the antibody titre to decline with time, and fourthly, two sows (A1 and 278) barely affected their piglets even though their

antibody titres at farrowing were comparable with those that in other instances had rapidly killed piglets.

A few more observations were made on this herd following upon the last vaccination which produced titres of 1024 (twice), 4096 and 8000 against the Large White boar. It seemed possible that one of these four sows might yet produce severe haemolytic disease and all four were mated to the Large White boar. With sow 278 the titre had fallen to 128 in four months and one month later, at farrowing, the piglets were clinically normal. The titres in sows 314, A1 and B8 had also fallen after four months: to 1024, 1024 and 512, respectively. Sow 314 was the next to farrow (seven months after vaccination) and her piglets, as also those later of sows A1 and B8, were likewise normal. The titres at farrowing are not known.

Herd C. Our attention was directed to this herd of forty sows, mainly Wessex, when one sow lost nine of twelve pigs with haemolytic disease. The sera of a further sixteen sows were then examined for iso-antibodies. The serum titre of the original sow was 512 and among the other sows there were two (both Wessex) with high titres against the cells of the two Large White boars. The first had a titre of 1024, and although it had recently farrowed to the same boar and had not been vaccinated since farrowing, it was stated that the litter had been normal after birth and successfully reared. The second sow had farrowed a litter to one boar whose cells gave a titre of 128. She was now in pig, however, to the other boar to whose cells the serum titre was 2048. It seemed possible, therefore, that these two animals might endanger their piglets and the subsequent litters of both were studied in detail.

The first sow farrowed twelve piglets all of which became severely affected with haemolytic disease. The haemoglobin levels were almost uniformly reduced to a mean value of 3·9 g./100 ml. but the whole litter survived. It is probable that the previous litter had been similarly affected on the farm and that this had been unobserved. The sow has since farrowed twice more to one of our own boars without further vaccination; the first litter was severely affected (with one death), the second was completely destroyed by the disease. The second sow farrowed twelve piglets, five of which were born dead. The remainder were severely affected with the disease and only three piglets survived. In this herd, therefore, the deductions based on the iso-antibody titres were in large measure fulfilled.

Herd D. That the disease may become a serious problem is illustrated by this herd of about one hundred and fifty, mainly Essex, sows in which several Large White boars were used. It was stated that over a period of four winter months, eleven litters had shown symptoms of jaundice (the first case occurring six months after vaccination) and whereas mortality had been heavy in only two instances, it was claimed that the piglets that did not die checked in growth so that the problem was nevertheless important. Seven of these animals were bled thirteen to twenty-six weeks after farrowing and in only one animal was the titre more than 8, namely 256. In each case the serum was titrated against the cells of the boar that sired the affected litter. A provisional diagnosis was made, therefore, of a non-haemolytic condition. However, when the sow above, with a serum titre of 256, farrowed again the litter was clearly affected with haemolytic disease and only four of the nine live-born pigs survived.

It was probable, therefore, that the low titres in the other sows had been higher at farrowing, and all further vaccination of the breeding stock was stopped.

During the next ten months it was reported that seven more sows had affected litters. These included only one animal that had had affected piglets before (the particular sow already discussed) and at this, her third farrowing (fifteen months after vaccination), all eleven pigs died rapidly with severe anaemia. One of the other sows was not examined at farrowing, but as the serum titre was less than 4 two weeks later it is very doubtful whether the condition was haemolytic disease. Another litter was clearly unaffected clinically and the piglets' cells were only very weakly sensitized; but the litters of the remaining four sows were obviously affected, three piglets dying in one litter and six in another.

The findings in this herd remain a little unsatisfactory, mainly because the high incidence of the disease was not observed entirely at first hand. It has been claimed that seventeen different sows had affected piglets. Although only six of these were examined at farrowing by one of us, in five litters the disease was obviously present. In addition, we have personally established that at least forty-six piglets died of haemolytic disease during a period of fifteen months.

Herd E. This herd of about one hundred and twenty Essex sows and four Large White boars demonstrated well a further pattern of the disease as it occurs in the field. The herd had been vaccinated last on December 2nd. Between January 12th and 18th four litters were affected with haemolytic disease. One litter was lost completely, the second lost six out of ten pigs (and two other piglets were saved probably because they were transferred to another sow), the third was severely affected but all the pigs recovered, and the fourth litter was affected but not severely. Twenty-one other sows were bled and although seven of these had titres of 512 or more, it was reported later that their litters were unaffected. Furthermore, when the original four sows farrowed again no signs of the disease could be seen by the owner. Serum samples were obtained from two of these latter sows after the second farrowings and it was found that their antibody titres had declined from 256 and 512, respectively, to 32 in each case. It seems likely, therefore, that in this herd the antibody titres had generally declined, so that only the sows that farrowed shortly after vaccination had had affected litters.

Influence of Vaccination, Iso-antibody Titre and Breed

The diagnosis of haemolytic disease is by our definition a laboratory procedure. When iso-antibodies are common and piglet mortality is extensive, however, laboratory findings alone cannot be used to decide the cause of death (Goodwin, 1954). This was not appreciated during the very early work on this disease but all the cases discussed in the present paper have been subject to more stringent diagnostic criteria. The most reliable diagnosis of the serious forms of the disease is undoubtedly made clinically, and from a consideration of all the clinical evidence together with the supporting serological findings, thirty-two of the sows that have now been investigated by one of us can be accepted as having had litters that suffered markedly from haemolytic disease. These cases, which

came from eighteen herds and include twenty-four litters where the disease was fatal, are analysed below.

Vaccination. Of the thirty-two sows with clinically affected piglets, thirty had definitely been vaccinated with crystal violet vaccine. Twenty-six had been vaccinated twice or more and three others known to have been vaccinated once could have been injected more often. One animal was said to have been vaccinated once only and detailed enquiries suggest that this may be so. It was maintained that the remaining two sows had not been vaccinated throughout their breeding life on the farm where they were examined but they were both bought as in-pig gilts from a farm where vaccination was practised. As the original owners could not state whether these two animals had been vaccinated, no animal known to us has had clinically affected piglets and yet been clearly unvaccinated. The vaccination records are neither numerous enough nor sufficiently reliable in defining the frequency of vaccination above twice to analyse them statistically, but the present evidence suggests that vaccination above twice does not increase the risk proportionally. It appears that the second vaccination usually brings the iso-antibody titre to a level that is representative for the animal; subsequent vaccination does not increase the titre markedly although it may restore a falling titre to approximately its original value (see Table 1).

When the sow's clinical history departs from the classical one of normal farrowings before the second vaccination with affection of successive litters thereafter, a simple explanation is usually available. Thus some animals have lost piglets with the disease after the second vaccination, but have not continued to destroy their litters either because the antibody level has fallen or because the boar has been changed. In a few instances a litter has been reared after the second vaccination and then later, without further vaccination, a litter has been seriously affected or lost. In these cases the affected litter was sired by a new boar. There have been three sows, however, whose histories are not at once explained by existing knowledge. In the first, the sow was vaccinated only once and then had three litters by the same boar, the first two litters being successfully reared. The third litter was obviously affected and three out of ten piglets died on the sixth day. A possible explanation may be as follows. The disease in the third litter was only just lethal, death at the sixth day being about the limit before recovery, and the previous litters might well have been less affected and, consequently, overlooked. It is known that the antibody titre can fluctuate and increase in the absence of vaccination (Fig. 1) and such an increase might have occurred before the third farrowing. The other two cases were those already mentioned as being unvaccinated by the owner although bought from a vaccinated herd. They had been on their present farm for two years and each had had two previous litters

by the same boar without any apparent trouble. The fact that they had affected litters together suggests some common stimulation.

Iso-antibody titre. The serum iso-antibody titres obtained with the direct agglutination and indirect sensitization tests are listed in Table 2. They refer only to those sows where the disease was fatal. Two of the twenty-four such cases cannot be included as the boars that sired the affected pigs had been slaughtered. It can be seen that the disease has not usually been fatal when the serum titre of the sow was less than 512 (I.S.T.).

TABLE 2

ISO-ANTIBODY TITRES

Twenty-two sows that lost piglets with haemolytic disease.

Sow	*Titre by*		*Sow*	*Titre by*	
	D.A.	*I.S.T.*		*D.A.*	*I.S.T.*
1	32	2048	12	128	1024
2	8000	32,000	13	128	1024
3	32	1024	14	*	>512
4	128	8000	15	*	512
5	16	1024	16	*	>512
6	8	1024	17	*	4000
7	4	512	18	*	8000
8	64	512	19	*	32,000
9	16	1024	20	*	>512
10	256	2048	21	*	1024
11	128	1024	22	*	2000

*Titration not performed.

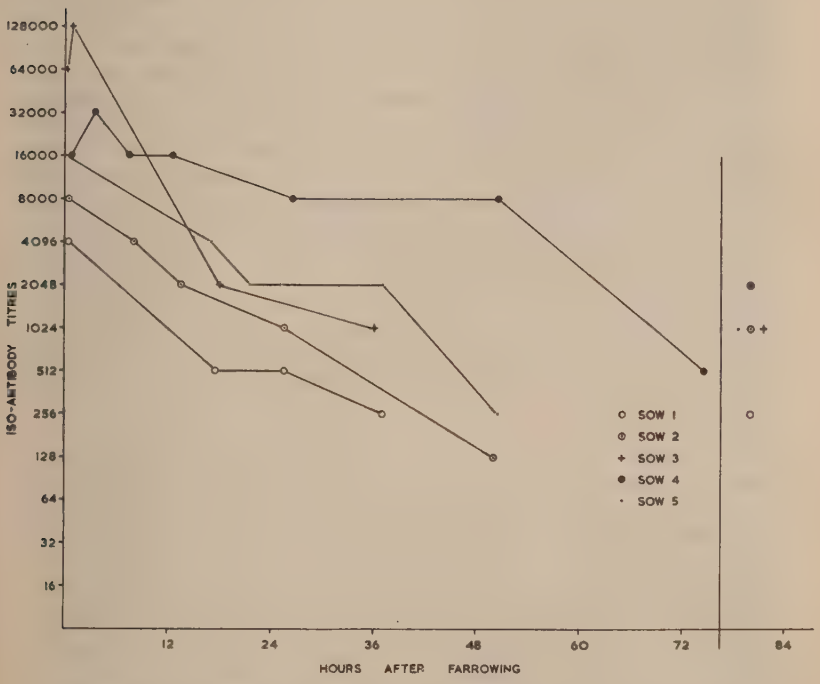
Breed. Twenty-six of the thirty-two clinically affected litters were from Essex/Wessex x Large White matings. The remainder were from Essex x Landrace (2), pure Wessex (2), pure Essex (1), and pure Landrace (1) matings. Recently the disease has been reported in pure Tamworth pigs but the diagnosis is still in doubt. Thus of the substantiated cases, there was only one where the sow was not of the Essex/Wessex breeds.

Changes in the Colostral Iso-antibody Titre and the Permeability of the Gut after Birth, and their Effect on the Course of the Disease

Although the different clinical effects produced in different litters by the same serum iso-antibody titre may be due to the fact

that complicated mixtures of iso-antibodies are titrated together, consideration has been given to the ability of different sows to concentrate antibody in the colostrum. Fig. 3 shows the colostral antibody titres and the serum antibody titre at farrowing for five sows. All the values were obtained with the indirect sensitization test. The first colostrum sample was taken during farrowing in

Fig. 3.



Post-farrowing change of colostral titre in five sows.
Note: The serum titre at farrowing is shown by the isolated symbol in each case.

every case. It can be seen that the initial concentration and subsequent decline of antibody in the colostrum was broadly similar in all the animals. During farrowing the titre was substantially higher in the milk than in the serum but by twenty-four hours later the colostral titre in four sows was comparable with that in the serum at farrowing. The fate of a litter is not primarily determined by the degree of colostral concentration of antibody (Fig. 3). Sow 5 rapidly destroyed her litter and sow 4 produced only moderate clinical signs; yet sow 4 concentrated iso-antibody to the same extent as sow 5. Furthermore, sow 3, whose colostral antibody titre at farrowing was about 128,000, did not produce such a severe

form of the disease as sow 1 where the corresponding titre was 4096 and the litter died rapidly. A similar conclusion was reached after observing the colostral antibody titres in herd B (second section of these results): the colostral iso-antibody titre in sow A1 twelve hours after farrowing was equal to that in sow 1 (Fig. 3) at the same time, while the serum and colostral titres of sow 278 were equal at about twelve hours after farrowing and this implied that the survival of the piglets could not be ascribed to a low concentration of antibody in the colostrum.

The colostral titre was also studied to see whether the decrease in antibody level after farrowing was sufficiently rapid to account for variations in the severity of the disease within a litter. The changing permeability of the neonatal gut was observed for the same reason and it was hoped that the knowledge gained on these two processes might allow piglets to be saved in the field. The permeability of the gut was studied mainly by preventing piglets from suckling at birth and returning them to the sow at increasing time intervals. It was necessary to choose litters that would not quite die of the disease and in these cases antibody has been found on the red cells after suckling when the period away from the sow was as long as thirty hours. However, when direct sensitization tests were made on the red cells of several pigs in a litter that had been successively returned to the sow it was found that the degree of sensitization decreased markedly and progressively in proportion to the length of time that the piglets had been separated. Yet the extent to which the haemoglobin concentration was reduced was not so markedly different. A similar uniformity in the degree of anaemia despite the staggering of the suckling periods was observed by Goodwin, Hayward, Heard and Roberts (1956). It appeared from these limited observations that the severity of the disease could not be greatly modified by restricting the suckling time during the first twenty-four hours and this has been confirmed in practice when dealing with acute cases of the disease. As piglets cannot be removed from the sow for longer than about twenty-four hours after birth before death ensues from other causes, the only other method would be to prevent all suckling for about twenty-four hours; this has likewise proved ineffective and it would appear that there is often much more antibody available than is necessary to kill piglets. It is not doubted that piglets could be saved in some litters if farrowing were attended and each piglet's suckling time were carefully and severely restricted but the procedure is not a simple one that can be easily practised under farm conditions. It may be deduced from these practical and experimental findings that only very slight differences in the severity of the disease would occur as a result of changes in the colostral titre and gut permeability in a litter suckling normally and born in a space of a few hours. Hence the explanation of the not uncommon fact that some piglets can be very severely affected while their litter-mates are almost normal, may well lie elsewhere.

Incidence of Sensitized Cells within a Litter

On sampling a litter of pigs for sensitized cells it was commonly observed that if one animal gave a strongly positive result with the direct anti-globulin sensitization test then the remainder of the litter did so likewise. This generalization applies only when the cells are strongly sensitized; for when the D.S.T. results are weak the cells of some piglets in the same litter often appear to be unaffected. This is understandable if the sensitization test is working at the limits of its sensitivity, and if, in addition, the degree and duration of sensitization vary slightly among the litter. To check this impression of total affection within a litter more precisely, a large series of routine diagnostic tests (D.S.T.) have been analysed. Within this series of unselected and consecutive tests there were eighteen litters where at least one piglet had strongly sensitized cells. Altogether, one hundred and thirty pigs had been sampled from these eighteen litters, in eight instances the whole litter being bled but in the remainder only a proportion of the litter (never less than three piglets). Only one of these one hundred and thirty pigs possessed unsensitized cells and this particular animal, being very small and weak, did not suckle for long, if at all. In our experience, therefore, haemolytic disease usually affects all the pigs in a litter. Should an exception be found to this, the first consideration must be whether the animal concerned suckled adequately.

DISCUSSION

In the light of the foregoing results, together with other observations that have been made during the course of this work, it is now possible to summarize the main influences in the epidemiology of haemolytic disease in the new-born pig.

The disease is consequent upon vaccination with crystal violet swine fever vaccine. One injection usually sensitizes the sow and little iso-antibody is produced. In a few instances, however, a single injection is capable of stimulating a high antibody titre which, in turn, may kill piglets. The ability of animals to respond to subsequent injections varies greatly and with the present vaccine there is a breed difference in this respect (Goodwin and Saison, 1956) so that sows of the Essex/Wessex breeds appear more at risk than Large Whites. Even among Essex/Wessex sows, however, many animals seem to produce a titre that is innately characteristic, which suggests that the extent of the response is largely predetermined in a herd. The second injection is usually critical and while some animals do not affect their piglets seriously until after the third injection, many do so when they have been vaccinated twice. The antibody titre rises steeply after the second vaccination and reaches a peak in about two weeks. It may then decline, in which case the risk is maximal shortly after vaccination, or it may persist for months or years at a fairly steady level. In the latter cases unexplained

fluctuations may be superimposed upon the persistent base-line titre.

If the serum iso-antibody titre is 512 or more against the boar's cells by our techniques there is a risk that piglets may die of haemolytic anaemia. The titre itself is not a reliable prognostic aid; some animals with a titre of 512 can produce an extremely acute form of the disease while others with the same titre barely affect their offspring. Thus a sow with a titre of 32,000 at farrowing lost only one out of seven pigs with haemolytic anaemia; and this piglet did not die until the fourth day. These anomalies merit further study although their solution may be advanced by the blood-group studies now in progress.

Although a litter is sometimes reprieved by changing the boar, among Large White boars an alternative sire is very commonly without benefit (Goodwin and Saison, 1956). It is probable that some of the litters whose survival has been attributed by the owners to a new boar were, in fact, no longer in danger because the antibody titre had fallen. The incidence of the disease is governed mainly by the varying antibody response of the sow population. It is common for a whole litter to be destroyed by the disease. When only some piglets die, however, the remainder have been found to possess sensitized cells.

In passing from a consideration of the epidemiology to an assessment of the economic importance of the condition, rather more doubt arises. The disease as defined by sensitized cells is widespread among vaccinated herds, but this is often of little practical importance. On the other hand, there are several reasons why the mortality from this condition cannot be deduced from the number of fatal cases that we have investigated during a period of two years. First, it is well known that cases of a new disease rarely accumulate rapidly. Secondly, all but two of our cases have derived from farms in the Cambridge area of the country; this argues that cases are being overlooked elsewhere. Thirdly, many cases have been brought to our notice very fortuitously, mainly when piglets have been subject to a routine post-mortem examination, and only rarely has a case been notified primarily as haemolytic disease by a practitioner. It is clear that few practitioners are recognizing the condition. Fourthly, eighteen of the clinical cases have arisen in a total of only five herds and it is unlikely that among the many vaccinated herds of the country there are not others basically similar to these.

It is important to note that haemolytic disease in the new-born pig is not the result of a particular type of mating which, occurring spasmodically, results in mortality. The acute clinical cases are arising from an extensive subclinical background. That most cases are subclinical is, in retrospect, fortunate; for few workers would have been prepared to predict that the pig population could weather so successfully the repeated injection of what is, in effect, bulked porcine blood.

CONCLUSIONS

A study has been made of the epidemiology of haemolytic disease in the new-born piglet with special reference to the incidence of the condition in the field.

It was observed that, in some animals, once the antibody titre had been brought to a high level it remained high for several years and thus successive litters of piglets became affected with the disease. As yet unexplained fluctuations in titre were superimposed upon the basic high antibody level. In a group of pigs that were vaccinated experimentally, however, the antibody titre was not maintained; instead, it reached a peak shortly after vaccination and then declined steadily. These two types of antibody response were commonly observed among vaccinated sows in the field.

To observe the incidence of haemolytic disease in vaccinated herds, five such herds, including two that had no history of the disease, were studied in detail. The incidence of litters with sensitized cells was lower than expected because in some sows the antibody titre declined with time. Nevertheless, several litters had sensitized cells and some were affected with varying degrees of anaemia and jaundice. It was noteworthy that commonly the piglets could be brought close to an irreversible degree of anaemia without attracting attention. Severe cases of haemolytic disease could not be forecast from a knowledge of the antibody titre in the sow: although some of the sows that appeared to be at risk eventually lost piglets or had litters that were severely affected, there were others where a high antibody titre had little ill-effect.

Records are available for thirty-two sows where the litters were clinically affected with haemolytic disease. Thirty of these animals had been vaccinated with crystal violet vaccine; with the remaining two the vaccination history was uncertain. In most cases two or more injections of vaccine were necessary before the disease became apparent.

With twenty-two sows where the disease was fatal and the sow's serum was titrated against the sire's cells, the iso-antibody titre close to farrowing was 512 or more by our techniques.

The disease has been seen mainly in piglets from the Essex or Wessex x Large White cross but it has also occurred in pure Essex, pure Wessex and pure Landrace piglets. In only one case was the sow not of the Essex/Wessex breeds.

Differences in the degree of affection between litters could not be explained on the basis of marked differences in the ability to concentrate iso-antibodies in the colostrum. Neither did the colostral titre decline rapidly after farrowing so that suckling could soon be permitted with safety. Relative to this question, attempts to preserve a few litters in the field by limiting their suckling time have been, in the main, unsuccessful.

Following normal suckling. the presence of strongly sensitized

cells in one piglet of a litter has invariably been associated with sensitization throughout the whole litter.

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CONTAGIOUS PUSTULAR DERMATITIS VIRUS*

I. Studies on Morphology

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INTRODUCTION

A direct study of the infective agent of contagious pustular dermatitis (C.P.D.) has not been attempted before the present inquiry, except for a single experiment of Borcila and Isopescu (1937) who tried to stain it with victoria blue in a smear of vesicular fluid. As will be shown later, they failed to demonstrate the virus. In the present work, the method of differential centrifugation was adapted to obtain relatively pure suspensions of elementary bodies (e.bs.) of the virus and it was possible to study their staining characters and morphology under the ordinary and electron microscopes. Reference will be made to the importance of these observations on the diagnosis of the disease.

MATERIAL AND METHODS

Strains. The two strains of the virus used were 'U.F.' and 'W', both of English origin. The former isolated by Glover (1928) from sheep at Cambridge in 1927, has been maintained in sheep. The 'W' strain isolated from a human case of the disease in Woldingham, Surrey, in 1947, was also maintained in sheep. Each of these strains showed a similar biological activity and morphology.

Preparation of elementary body suspensions. The technique of differential centrifugation commonly employed for vaccinia virus (Parker and Rivers, 1935; Bennett, Horgan and Haseeb, 1944) was followed with some modifications. The virus was propagated on the skin of lambs under one year of age and reared on C.P.D.-free farms. The best time of harvesting the virus was found to be the fourth day after inoculation by scarification, when vesiculation, as evidenced by greying of the surface of papules which appear first on the third day, was complete. Material harvested on the third day yielded poor suspensions and that from the sixth day onward yielded some non-specific particulate matter which interfered with the purity of the suspension. Poor e.b. suspensions with much inactive particulate matter could, however, still be prepared from scabs on the 18th day after inoculation.

The suspensions remained in a dispersed state for several weeks, after which small clumps of about 10 to 20 e.bs. began to form and sediment.

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However, a considerable number of e.bs. in a one-year old suspension were still in a dispersed condition. The viable aerobic bacterial count was generally below 300 organisms per c.c. The e.b. mass sedimented in the course of the washing was brownish-white in colour. When brought into suspension and adjusted to an opacity between Brown's tubes 4 to 6, it was milky in appearance.

Attempts were also made to prepare e.b. suspensions from the rabbit skin. The susceptibility of this animal being relatively lower than that of sheep, only poor suspensions with much non-viral material were obtained. Elementary bodies could not be identified with certainty in suspensions prepared from the infected chorio-allantois of the chick embryo.

Smears of lesions. In smears of skin lesions, e.bs. could generally be demonstrated satisfactorily at any stage before pustule formation. The surface was scraped with the sharp edge of a scalpel and smeared thinly on a clean slide. The clarity of such preparations increased markedly if the air-dried smears were placed vertically in distilled water for ten minutes and thereafter dried again before fixation. The techniques of staining and electron micrography are best described along with the results and will be found in the appropriate sections which follow.

RESULTS

Unstained Elementary Bodies

Under dark-field illumination, fresh e.b. suspensions showed a large number of dispersed, uniform, round particles which scattered light evenly without a brighter boundary such as is shown by bacteria. They showed active Brownian agitation which, at 15 to 20° C., comprised a swing of more than the diameter of the particle itself. If such a preparation is kept at 0 to 4° C. for a few days, the e.bs. settled on the surface of the slide or the cover-glass in small clumps, short chains, pairs or singly. The diameter of the uniform particles measured approximately 0·3 μ. After several weeks old preparations showed larger clumps which scattered a large amount of light. Individual particles were noticeably reduced but still present in considerable numbers.

Although dark-field was the only satisfactory method of examining the living virus and could be carried out with speed and facility, it supplied only limited information and was not as suitable as the staining technique for studying smears of lesions.

Staining Characters

In the present investigation, methods reported to have been successfully employed in staining e.bs., particularly those of vaccinia and variola, were applied to C.P.D. virus. Several variations and modifications were also tried and often the procedure was controlled by including in the preparations viruses of known staining properties. It will be seen from what follows that the method of Paschen (1906, 1917) gave the best and most consistent results.

Paschen's method. This method was found suitable for staining the e.bs. of C.P.D. in smears of washed suspensions and skin lesions of

sheep as well as rabbits. The technique of Paschen (1917) and van Rooyen and Illingworth (1944) was suitable. By experience, however, it was found that carbol fuchsin mixture aged for 1 to 12 months stained the e.bs. quite deeply and the background considerably lighter than when a freshly-mixed stain advocated by the joint authors was used.

The e.bs. appeared as deep-red, round structures of strikingly uniform shape against a roseate background. Both in e.b. suspensions and smears of skin lesions they were usually very numerous and disposed singly, in short chains of up to 4, or in small clumps. The background was usually deeper in lesions but there were many thin areas in the smears spread with a scalpel. The stained e.bs. measured between 0·2 and 0·3 μ.

Herzberg's method. Victoria blue 4R was used for staining e.bs. of many viruses in smears and inside tissue cells by Herzberg (1934, 1936a, 1936b). Borcila and Isopescu (1937) employed this method to stain the C.P.D. virus in trypsinised smears of vesicular fluid. In the present investigation, their work was repeated and it is believed that they failed to demonstrate the virus. In general, victoria blue 4R was found to be a poor stain for the virus and faint staining was obtained only after a long contact. Various modifications of the method failed to improve the staining.

The method of Gutstein (1937), in which methyl violet is used, was unsatisfactory since only a faint bluish-violet staining was obtained which was of little practical value. Nevertheless MacDonald (1951) has since reported good staining with this method.

Control skin-lesion smears, untreated or trypsinised, showed numerous e.bs. when stained by Paschen's method. On the other hand smears stained by the method of Herzberg or modifications thereof showed punctiform deposits consisting of minute roundish deposits rather uniformly distributed over the smeared as well as unsmeared areas of the slide and of a colour varying from light bluish-violet to dark blue and black. Their distribution and numbers were quite different from e.bs. The latter were also much more uniform in shape and depth of colour. The punctiform spots were, therefore, considered to be artefacts and it is believed that Borcila and Isopescu (1937) saw only these spots in their smears.

It will be seen from the foregoing experiments that victoria blue 4R stains the C.P.D. virus only faintly after prolonged contact, the extent of which is limited to about two hours because, after that, the stain begins to deposit. The faint staining obtained is not of sufficient depth to be of practical value.

Morosow's method. Morosow (1926) adopted Fontana's method for staining the e.bs. of pig-pox and other viruses. In this study, washed e.bs. of C.P.D. stained a deep brown against a yellowish brown background. Each of the dark brown e.bs. showed a lighter brown ring around it which merged into the still lighter background.

This made the particle look slightly bigger than its real size. In infected tissue smears, however, the e.bs. could not be easily distinguished from other granular material, especially in the thicker parts of the smears. For this reason, Morosow's method could not be employed to the same extent as Paschen's method.

Other methods. Staining by Giemsa as described by Bland and Robinow (1939) was followed and later the modifications of Coles (1935) and Craigie (1933) were tried on washed e.b. smears as well as tissue smears. In all cases, the e.bs. entirely failed to take up the stain. Other methods tried entirely without success were the following: Machiavello (1937) and its modifications by Begg (1944), Fulton and Van den Ende and Fulton and Joyner (1945); the mercurochrome-methylene blue method of Bedson (1947); and common bacterial stains, such as Löffler's and one per cent methylene blue, one per cent aqueous eosin, strong and dilute carbol fuchsin (heated and unheated), and one per cent neutral red in water.

Electron Micrography

Technique. The mounts for the electron microscope were prepared by placing a small drop of the suspension on collodion-covered wire grids. After a few seconds the suspension was sucked off from the side with a fine capillary tube or the edge of a filter paper. Two to three minutes afterwards the grid was washed by dipping a few times in distilled water. It was found that this procedure did not dislodge the e.bs. even when the washing was more vigorous, and greatly improved the preparation by removing washable matter. The washed mount was dried over calcium chloride for half to one hour or more before examination. Some of the preparations were transferred to a thin film of beryllium by volatilising the metal in a vacuum and depositing it in a vertical direction on the specimen. The collodion was then dissolved away by placing the specimen in acetone for 30 minutes. Such beryllium-supported films were free from the disturbing effect of the thick collodion background and gave noticeably clearer pictures. For a 3-dimensional study of the virus, some of the collodion mounts were coated with gold deposited at an angle of 15 degrees, according to the method of Williams and Wyckoff (1945). It was found that thin gold deposits (ca. 8 to 10 Å) showed the surface structure and the thickness of the e.b. better than thicker deposits (ca. 80 to 100 Å). The latter, however, produced more vivid pictures with improved contrasts.

Most of the work was done with the R.C.A. Electron Microscope Model E.M.B.; some pictures were also taken with a Siemen's microscope. Many varying magnifications and penetrating powers of the electron beam were tried but the best results were obtained when an electron picture taken at a magnification of 7,000 to 10,000 (50 K.V.) was subsequently enlarged 3 to 4 times in an ordinary photographic enlarger.

An attempt was made to clear the internal structure of the e.b. by osmic fixation and by the action of enzymes, trypsin and pepsin. E.b. smears on the collodion film were exposed to osmic vapour for ½, one and two minutes and washed in distilled water. For trypsinisation, a small quantity of the e.b. suspension was mixed with an equal quantity of a one per cent solution of trypsin, previously extracted with ether to

remove lipids (Pirie, 1935) in 4 per cent anhydrous sodium carbonate and filtered through a Berkefeld V candle. The mixture was incubated for 1, 4, and 18 hours, washed several times in the angle centrifuge and examined. For pepsinisation, the suspension was mixed with an equal quantity of one per cent crystalline pepsin dissolved in McIlvaine's buffer (pH 3·0) and incubated for one hour. A part of this suspension was examined after washing on the grid; the rest was left in pepsin overnight at room temperature and examined after washing on the next day.

Structure. The appearance and dimensions of e.bs. in micrographs of the two strains examined were identical. As in stained preparations, the e.bs. were disposed singly, in pairs, in short chains or clumps of varying sizes according to the age of the suspension. Their disposition in relation to each other did not show any regular arrangement and they seemed to have come together in a random fashion. Neighbouring e.bs. were frequently joined by strands of a material of low electron absorbing power, which gives the impression of being a sticky coating on the surface. Whether it is really so or represents structural continuity, is not known.

In un-shadowed or conventional micrographs, the majority of e.bs. appeared as monomorphic short rods with rounded ends and resembling a small bacillus (Fig. 1). Some pictures showed nearly circular images of a noticeably greater density than the rectangular forms and having a diameter equal approximately to their width. These images were taken to be rods standing on end, an interpretation subsequently confirmed in gold shadowed micrographs where they were seen to throw a longer shadow, as expected in that position. Ovoid images with a vague shadow on one side of the long axis of the oval were taken to be optical sections of e.bs. in a slanting position.

The e.bs. did not show an internal structure with the same regularity as do those of vaccinia (Green, Anderson and Smadel, 1942). Most were uniformly dense but some showed round or half-moon shaped areas with a higher absorption of electrons in the subpolar region. The boundaries of these areas were not clearly defined and they merged into the surrounding parts which did not absorb electrons to the same extent. In some, a small round spot at or near the centre of the e.b. also showed higher absorption. This spot was, however, comparatively much smaller and rarer than the central spot in vaccinia, molluscum contagiosum and fowl-pox e.bs. (Macfarlane and Dawson, 1948; Ruska and Kausche, 1943; Lépine, Atanasiu and Croissant, 1949). Efforts were made to increase the definition of these internal structures by fixation in osmic vapour and treatment with trypsin and pepsin. Osmic fixation increased the absorbing power of the whole e.b. and obscured all internal structure. Trypsin and pepsin treatment, such as clears vaccinia e.b., entirely failed to have any effect. Prolonged pepsinisation disintegrated the e.b. without clearing the internal structure (Fig. 2).

Micrographs of gold-shadowed preparations failed to show any

marked degree of flattening of the e.b. as seen in vaccinia and other viruses (Beard, 1948). The length of the shadow was such as would be expected of round or slightly compressed cylinders with rounded ends (Fig. 3). This slight compression may have been caused by drying but was never so much as in vaccinia. The surface of the e.b. showed fine granular structures of varying sizes but no regular pattern or arrangement. There was also a suggestion of two swellings or raised areas corresponding with sub-polar densities observed in unshadowed micrographs. Because of these swellings, the side shadow thrown by some e.bs., on a plain surface was saddle-shaped, with two peaks. The central dense spot did not cause a surface swelling.

A few of the photographs taken with the Siemen's electron microscope showed the appearance depicted in Fig. 4. Appropriate tests with the microscope showed that this appearance was not attributable to diffraction, as a casual examination would suggest. This appearance was seen in micrographs taken at 50 K.V. as well as at 90 K.V. The fact that the e.bs. in this picture appear more compressed also suggests that the effect may be ascribable to damage in the electron beam.

The background showed small accumulations of vague material with low but varying capacity for absorbing electrons, resembling the bridging strands between closely placed e.bs. and being more frequent in or near large e.b. clumps. It is highly probable that this material was derived from the surface of e.bs. In this connection, it may also be mentioned that the buffer solution in which e.bs. of C.P.D. had remained suspended for some weeks developed an opalescence which could not be removed by centrifugation in the angle centrifuge. This hazy buffer solution from which e.bs. had been removed, however, reacted specifically with C.P.D. immune serum.

Dimensions. In conventional micrographs, the e.bs. measured from about 185 to 310 mμ in length, the mean being 251·8 mμ with a standard deviation of the mean of $\pm$1·57 mμ. The standard deviation (δ) was 22·55 mμ and the coefficient of variation (100 $\times$ δ/mean), 8·93 per cent. The breadth at the widest point measured 120 to 210 mμ, the mean being 158·18 mμ with a standard deviation of the mean of $\pm$1·23 mμ. The standard deviation (δ) was 17·66 mμ and the coefficient of variation 11·2 per cent. The axial ratio (length/breadth) varied between 1·27 and 2·03, the mean being 1·6 with a standard deviation of the mean of $\pm$0·0116 and the standard deviation of 0·166. Frequency distributions of length and breadth in groups of 20 mμ were normal as shown in Figs. 5 and 6. A scatter diagram of the length and breadth of e.bs. is given in Fig. 7. It will be seen that there are no distinct divisions of the scatter in lower or higher breadth groups and the diagram gives the impression of normal variation of a monomorphic organism. The variation of breadth at higher lengths seems to be slightly more, though the

Fig. 5.

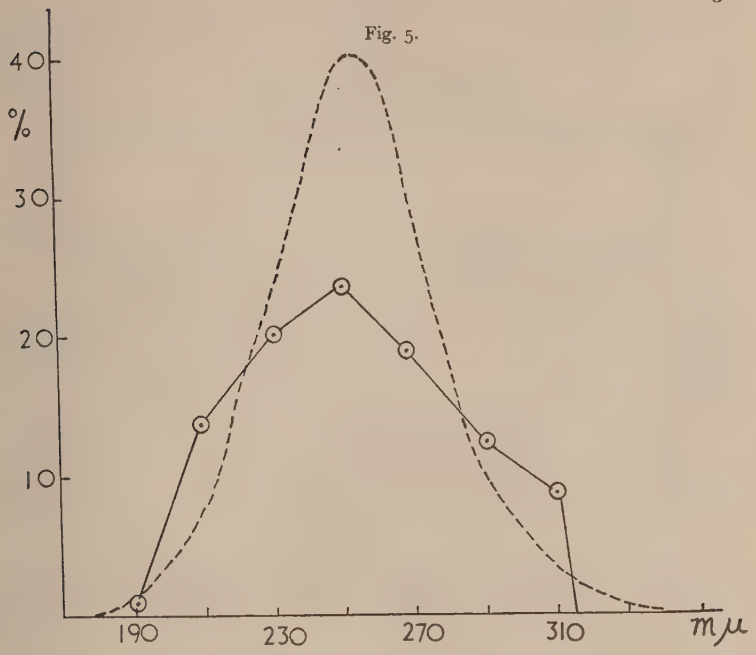


FIG. 5. LENGTH GROUPS

Fig. 6.

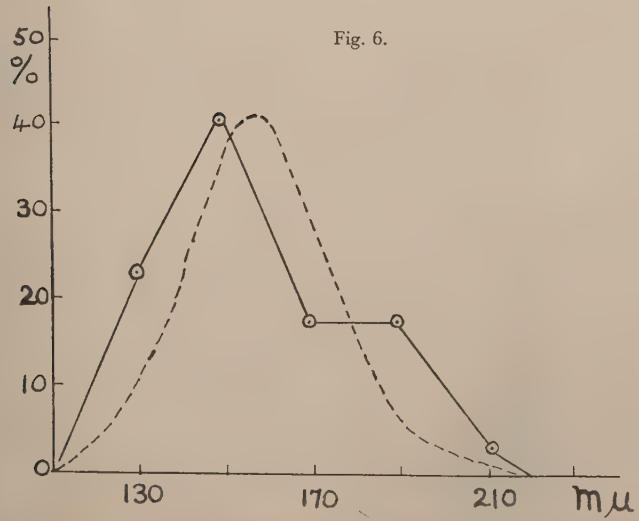
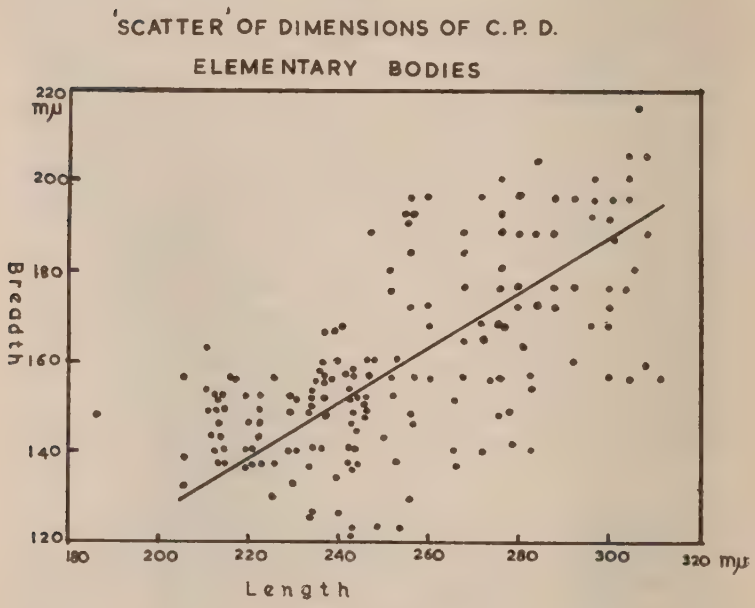


FIG 6. BREADTH GROUPS

Figs. 5 and 6. Frequency distribution of C.P.D. elementary bodies in 20 mμ groups. Calculated normal frequencies are shown in dotted lines.

Fig. 7.



'Scatter' of dimensions of C.P.D. elementary bodies.

variation of length at different breadth levels seems to be more or less uniform.

Dimensions of the electron images of some pox viruses and other related ones are put together for comparison in Table 1. Figures for viruses other than varicella, pig-pox and C.P.D. are taken from Ruska and Kausche (1943). Groupé and Rake (1947) did not measure their micrographs of fowl-pox virus and the only unshaded picture in their paper has not got a sufficient number of clearly defined e.bs. for measurement and statistical analysis. The measurements of varicella by Nagler and Rake (1948) were not strictly comparable but were included.

It will be seen from Table 1 that the eight viruses listed can be distinguished from each other on the basis of size and axial ratio. C.P.D. virus is the most elongated of them all, with an axial ratio of 1 : 1·6. Further, from the viruses of vaccinia, molluscum contagiosum and fowl-pox it can be distinguished on the basis of internal structure. All these viruses have a sizeable central dense spot which often causes a bulge when the virus collapses. In the C.P.D. virus, however, there are two dense spots near the poles as is sometimes the case in bacteria (polar bodies). Also, this virus does not settle on the collodion films with the same degree of collapse as does vaccinia (Sharp, Taylor, Hook and Beard, 1946) and fowl-pox viruses (Groupé

and Rake, 1947). It is also less amenable to the action of proteolytic enzymes than vaccinia; its lower penetrability by stains has been demonstrated in the previous section of this paper.

TABLE 1

DIMENSIONS OF ELECTRONIC IMAGES OF SOME POX VIRUSES

(All measurements in mμ)

Virus	*No. of e.bs. measured*	*Arithmetical mean and standard deviation* (δ)					
		Length (a)	(δ)	*Breadth* (b)	(δ)	*Axial ratio* $Q=a/b$	(δ)
Molluscum contagiosum	150	255	61·3	178	41·8	1·45	0·143
Ectromelia	90	232	24·6	172	9·6	1·34	0·024
Myxoma cuniculi	81	287	20·7	233	19·0	1·22	0·122
Canary-pox	75	311	26·1	263	22·7	1·14	0·108
Vaccinia	70	262	28·2	209	23·9	1·25	0·124
Varicella	—	238	—	210	—	—	—
Pig-pox	24	323	68·2	240	46·6	1·35	0·31
C.P.D.	206	252	22·5	158	17·7	1·6	0·16

Control examinations. Skin scrapings of a lamb experimentally infected with another eruptive skin disease called "strawberry foot-rot" were centrifuged and washed in a manner described above for preparation of C.P.D. e.b. suspensions. Several electron micrographs of this material were examined without seeing anything resembling C.P.D. e.bs. (Abdussalam and Blakemore, 1948).

DISCUSSION

The use of staining methods has been proposed for the diagnosis of variola (van Rooyen and Illingworth, 1944 and others) but they have only recently been used for the diagnosis of animal poxes (Abdussalam, 1950; Blakemore and Abdussalam, 1956).

In the foregoing experiments, a number of stains successfully employed in staining vaccinia and other viruses failed to stain the e.bs. of the C.P.D. or did so only partially. In effect only two, viz. Morosow's and Paschen's gave satisfactory results. The former was useful in staining washed e.b. suspensions but in the presence of tissue particles the e.bs. were not easily distinguished. Paschen's method is, therefore, the only one which is of practical value in staining the virus in tissue smears. Before pustulation, it is not difficult to demonstrate the abundant e.bs. in stained slides. In the pustular

and scab stages, however, particulate matter derived from breaking down tissues makes it difficult to recognise the dwindling numbers of e.bs. Another limitation of this method is that while it differentiates the C.P.D. lesion from bacterial lesions, it does not distinguish it from other poxes such as sheep-pox, goat-pox, vaccinia, etc. In spite of these limitations the method can be a valuable aid in clinical and experimental work.

The electron micrographs show that the e.b. of C.P.D. is a short rod with rounded edges and is monomorphic. The visual impression of monomorphism is confirmed by the uniform variations in frequency distributions of the dimensional groups. This remark, of course, applies here to virus particles of a more or less uniform density, for the method of preparation of e.b. suspensions precludes appreciably lighter or heavier virus particles, if any, in the original tissue-suspension.

Blanc and Martin (1941) calculated the size of the virus from filtration experiments to be between 100 and 260 mμ. This estimate, on the whole, seems to be smaller than the size measured from electron micrographs, but the difference may be caused by flattening of the e.b. as it settles and dries on the grid.

Morphologically, C.P.D. virus falls in the "quadriform" group ("gequadriförmigen Virus-arten") of Ruska and Kausche (1943), in which they placed the viruses of molluscum contagiosum, ectromelia, rabbit myxoma, canary-pox and vaccinia on the basis of electron micrographic studies. They expected that the viruses of varicella, herpes and animal poxes would also fall in this group. Of these, the viruses of fowl-pox (Groupé and Rake, 1947; Lépine, *et al.*, 1949), pig-pox (Blakemore, *et al.*, 1956) and varicella (Nagler and Rake, 1948) have since been studied with the electron microscope and can be assigned to this group. The e.b. of herpes simplex has, however, been found to be slightly different inasmuch as its shape varies from a sphere to doughnut-like form (Coriell, Rake, Blank and Scott, 1950).

In the present investigation, no evidence of the multiplication of e.bs. by the simple process of growth and division, as in bacteria, was found. Although the polar regions were found slightly swollen in some individuals, this feature was never found to progress to a constriction in the middle or dumb-bell formation. If a process of elongation and division were in progress one would find that variation in length would be considerably greater than in breadth. This was not found to be the case as the coefficient of variation did not differ materially in the two cases (length, 8·93 per cent and breadth, 11·2 per cent). One may argue that the process of differential centrifugation employed in the preparation of e.b. suspensions would eliminate such differences if they existed among e.bs. in the original tissue suspension. This is, however, only partly true. It was found that the mass of the smallest e.b. measured was roughly less than half of that of the largest and a whole range existed between them.

Under such conditions, growing and dividing forms, if any, could not have been completely eliminated. It seems probable, therefore, that the e.bs. of C.P.D. do not divide by simple division, or if they did, this process was arrested before they were prepared for examination in this work. The work of Ruska and Kausche (1943) pointed to a similar probability in respect of the virus species examined by them.

Since this work was done, Ishii, Kawakami and Fukuhara (1953) have reported seeing ellipsoidal e.bs. measuring 200 to 250 mμ in electron micrographs of smears of pustular material. The size reported is within the range found in the present investigation but they have not given any details.

CONCLUSIONS

A method of obtaining washed elementary body suspensions of the C.P.D. virus by differential centrifugation has been developed.

Staining reactions of the washed elementary bodies and those in smears of early lesions of the disease were studied by systematic trials of staining methods commonly used in virus work and their several modifications. Paschen's method was found to be the most satisfactory for practical application in diagnostic and experimental work. Experience showed that fixation of smears in methyl alcohol, avoiding over-heating of the mordant and the stain, thorough washing away of the mordant and the use of an aged stain instead of a freshly mixed one as usually recommended, assured good preparations.

In general, the C.P.D. virus is less penetrable by stains and proteolytic enzymes than vaccinia and related viruses.

Electron micrographs of washed elementary bodies showed them to be short rods with rounded ends and dense sub-polar regions. They are morphologically allied to the elementary bodies of pox viruses from each of which they can be differentiated on the basis of dimensions, axial ratio and internal structure.

Statistical analysis of dimensional measurements showed no evidence of division by simple fission or of pleomorphism.

ACKNOWLEDGMENTS

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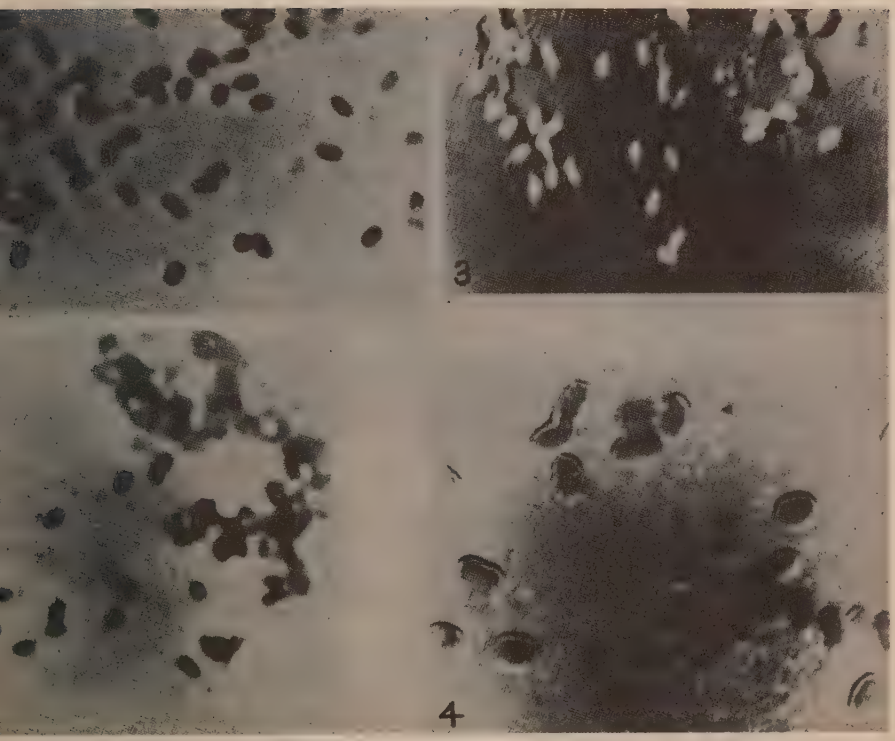

Fig. 1. Electron micrograph of osmic fixed smear of elementary bodies of C.P.D. virus. × 25,000.

Fig. 2. Elementary bodies treated with pepsin for 18 hours, showing disintegration without clarification of internal structure. × 25,000.

Fig. 3. Micrograph of a gold-shadowed preparation. × 25,000.

Fig. 4. Disintegration (?) of C.P.D. elementary bodies in the electron beam. × 36,000.

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STUDIES ON *CLOSTRIDIUM CHAUVOEI* VACCINE IN KENYA

By

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INTRODUCTION

Various workers have tested the ability of different media to support the growth of *Clostridium chauvoei* in the preparation of blackquarter vaccine. To prepare a new form of pellet vaccine, Roberts (1946) used a four per cent solution of Evans peptone in saline with 0·5 per cent glucose added. Rajagopolan (1947) produced a vaccine from cultures grown in glucose broth containing 0·05 per cent cysteine hydrochloride. A similar vaccine, when precipitated with potassium alum, successfully immunised sheep (Buddle, 1954). At Kabete, blackquarter vaccine prepared in meat piece medium proved to be satisfactory in the field (Piercy, 1951) but the preparation was costly and laborious. This paper describes the development, large scale production and field trials of a liquid blackquarter vaccine, using peptone water as the basic medium.

Although this experimental work is based on *Cl. chauvoei* as the major problem, *Cl. septicum* is not rare in Kenya and a combined *Cl. chauvoei* and *Cl. septicum* vaccine is prepared.

MATERIAL AND METHODS

Media. Varying concentrations of peptone in saline, containing 0·5 per cent glucose and 0·05 per cent cysteine hydrochloride (GCPW) were tested and compared with nutrient and "Lemco" broths containing the same additions.

Bacterial strains. The Konza-Kabete strain of *Cl. chauvoei*, a relatively avirulent strain, was used in the development and preparation of the vaccine. Stock cultures were kept in von Hibler's medium. Five ml. of an overnight culture failed to kill sheep and 1·0 ml. failed to kill guinea pigs when inoculated intramuscularly. A vaccine strain from South Africa, O.P. 64, and the L strain from Southern Rhodesia were also tested. The challenge strain, 1223, was isolated at Kabete in 1953 and its virulence maintained in horse meat medium (Piercy, 1951). The minimum lethal dose for sheep was 0·01 ml. of an overnight sub-culture in von Hibler's medium or GCPW, together with 0·5 ml. of a five per cent calcium chloride solution. The minimum lethal dose for cattle was difficult to ascertain, as known blackquarter-susceptible cattle were not readily available. However, six susceptible cattle were inoculated with doses varying from 5·0 ml. to 0·001 ml. of culture each containing 0·5 ml. of a five per cent calcium chloride solution: all died with typical blackquarter lesions. Two sheep controls inoculated with 1·0 ml. and 0·01 ml. also died. We assumed, therefore, that one sheep lethal dose of the challenge strain would kill cattle. The specificity of the Konza and 1223

strains was confirmed periodically by guinea pig protection tests, using specific *Cl. chauvoei* antiserum.

Preparation of washed bacterial cells. The bacterial cells of a 48 hour culture of *Cl. chauvoei* Konza strain, in GCPW, were centrifuged, washed in peptone water and recentrifuged. The deposit was suspended in GCPW to the original volume. The pH was adjusted to 7·6 and 1·0 per cent formalin added. After two days incubation, the pH was re-adjusted to 7·6 and 1·0 per cent potassium alum added. The mixture was incubated for four days and the pH finally corrected to 7·6.

Preparation of culture filtrate. A 48 hour culture of *Cl. chauvoei* Konza strain in GCPW was passed through a Ford's Grade S.B. Sterilmat. The filtrate was treated as the bacterial cells.

Adsorption. Aluminium hydroxide gel was freshly prepared by adding ten litres of a one per cent solution of ammonia to ten litres of one per cent ammonium alum solution. The gel formed was washed several times by decantation, filtered off and added to one litre of formolised culture. The pH of the culture had previously been corrected to 7·2.

Vaccine production (GCPW). Four per cent peptone and 0·5 per cent sodium chloride, dissolved in water and the pH adjusted to 7·8, was sterilised for 20 minutes at 15 lb. pressure. When required for seeding, the basic medium was filtered, glucose and cysteine added and the pH adjusted to 7·2 to 7·4: after resterilisation the medium was rapidly cooled and seeded when the temperature was approximately 37°C. It was found convenient to prepare twelve batches of medium each of five litres at one time. This yielded approximately 24,000 doses of vaccine. The recommended cattle dose was 2·5 ml.

Seed material. A tube containing 50 ml. of GCPW was inoculated with 0·5 ml. of *Cl. chauvoei* Konza strain stock culture, incubated at 37°C overnight and used to seed each flask containing five litres of medium. The same method of preparation was used for *Cl. septicum.*

Technique. Seeded medium was incubated at 37°C for 48 to 72 hours. The cultures were bulked in large containers, the pH was adjusted to 7·4 and one per cent formalin added. After 48 hours incubation the pH was re-adjusted to 7·4. The vaccine was precipitated with one per cent potassium alum and re-incubated for four days. The vaccine was then held at room temperature whilst sterility tests were carried out. When these proved satisfactory, the pH was again corrected to 7·4 and the vaccine bottled.

Toxicity tests. Merino sheep were preferred, but when they were not available, native East African fat-tailed sheep were used. Each batch of vaccine was tested in sheep, which were vaccinated subcutaneously. A satisfactory vaccine produced a minimal reaction at the site of inoculation to a dose of 2·0 ml. and gave complete protection to challenge.

Challenge. After 21 days, the vaccinated sheep and the controls were challenged intramuscularly with 1·0 ml. of an overnight culture of *Cl. chauvoei* strain 1223 in either GCPW or von Hibler's medium. Two days prior to each challenge, a preliminary titration of the virulence of the challenge strain was carried out, using whenever possible a standard inoculum of 1·0 ml. comprising the culture dilution in 0·5 ml. of broth plus 0·5 ml. of a five per cent solution of calcium chloride.

Biometrical methods. The yields were subjected to the analysis of variance (Snedecor, 1950). The potency and toxicity results were examined for independence by the chi-square test.

RESULTS

Experimental Findings

Growth in various media. The yields of dried bacteria, expressed as mg. per cent obtained in GCPW, nutrient and "Lemco" broths, in four comparative experiments, are given in Table 1. The growth obtained in GCPW was significantly greater than that obtained in the other media ($F = 4{\cdot}79$ for 2 and 7 degrees of freedom).

TABLE 1

GROWTH OF *CL. CHAUVOEI* IN VARIOUS MEDIA

Medium	*Growth expressed as mg. of dried bacteria per cent*			
	Exp. 1	*Exp. 2*	*Exp. 3*	*Exp. 4*
GCPW	85	69	73	80
"Lemco" broth	70	43		66
Nutrient broth		43	55	62

Effect of varying concentrations of peptone. Roberts (1946) used a four per cent concentration of peptone to prepare pellet vaccine and a similar concentration was used in our vaccine. Lower concentrations were found to affect the yield (Fig. 1). The relationship was linear over the range tested and significant at the one per cent level ($Y = 28{\cdot}4 + 13{\cdot}8X$, $r = 0{\cdot}95$).

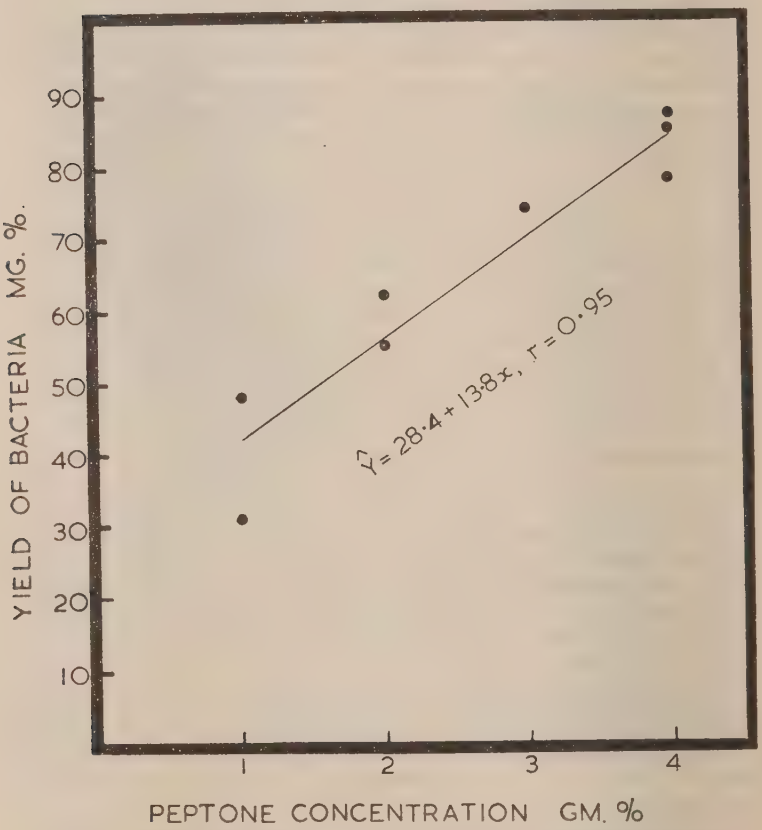
Effect of the source of peptone. The yield of *Cl. chauvoei* was not influenced significantly by the brand of peptone used ($F = 2{\cdot}44$ for 3 and 21 degrees of freedom.) The average yield of dried bacteria from 100 ml. of medium containing any of the four brands tested, was 78·8 mg. with a standard deviation of 1·8 mg.

Growth of different strains in GCPW. A profuse growth was consistently obtained in GCPW from any of the following strains of *Cl. chauvoei:* Konza, O.P.64, L and 1223.

Toxicity and potency tests. Blackquarter vaccine prepared from *Cl. chauvoei* Konza strain grown in GCPW occasionally produced minor local reactions in sheep, following subcutaneous inoculation. Twenty-nine batches of vaccine were tested in 37 Merino sheep and 32 batches in 83 native sheep. All vaccinated sheep of both races were immune to intramuscular challenge with the virulent strain 1223, but race differences were noted in the unvaccinated controls. The eight Merino controls and 13 out of 17 native controls died.

Nevertheless, the protection obtained in native sheep was highly significant (chi-square 65·85, $P<0.001$).

FIGURE 1.



The relationship between peptone concentration in the medium and the yield of *Cl. chauvoei*.

Keeping quality of vaccine. Vaccine, tested for potency and containing equal parts of *Cl. chauvoei* and *Cl. septicum* cultures, was kept at room temperature (18 to 23°C.) in the dark and retested after 6, 12 and 19 months storage at dosage levels varying from 1·0 to 5·0 ml. All the vaccinated sheep withstood challenge with 1·0 ml. of virulent *Cl. chauvoei* culture, which killed all the controls (Table 2).

Potency of washed bacterial cells and culture filtrate. A 48 hour culture of *Cl. chauvoei* Konza strain in GCPW was divided into three aliquots. The first was used to prepare "normal" blackquarter

vaccine, the second washed bacterial cells and the third culture filtrate. None of the sheep inoculated with culture filtrate survived challenge with 1·0 ml. of virulent *Cl. chauvoei* culture, whereas the sheep given the other vaccines were immune (Table 3). The number of sheep involved was small, but the difference between the potency of culture filtrate and the other two vaccines was apparently significant (chi-square 5·22, $P < 0{\cdot}05 > 0{\cdot}02$).

TABLE 2

EFFECT OF STORAGE ON THE POTENCY OF VACCINE TESTED IN SHEEP

Storage period (months)	*Vaccinated*				*Controls*
	Vaccine doses (ml.)				
	1·0	*2·0*	*4·0*	*5·0*	
0	—	0/5	—	0/5	4/4
6	0/2	0/3	—	0/3	2/4
12	0/2	0/4	0/4	—	2/2
19	0/2	0/2	0/2	—	2/2

Numerator = Deaths
Denominator = Number of sheep challenged.

Field Trials

History. Field trials were carried out on a farm considered by the owner to be heavily infected with blackquarter. The average annual mortality rate over the previous eight years was 14 ± 6 out of approximately 2,500 cattle at risk. The deaths had occurred in cattle whose ages ranged from calves to ten years. The longest period during which the farm was free from blackquarter deaths in that time was seven months. The practice was to vaccinate calves at four months and again at weaning: thereafter, all cattle were vaccinated every six months. The results following vaccination with certain commercial vaccines had been unsatisfactory. For example, 15 deaths had occurred within four months of the last vaccination in June, 1954.

Vaccination. Two vaccines were prepared from the same cultures. In one, the cultures were precipitated with potassium alum (UM vaccine). In the other, the cultures were adsorbed onto aluminium hydroxide (OH vaccine). Both vaccines contained equal parts of *Cl. chauvoei* and *Cl. septicum*. The dose was 5·0 ml. administered subcutaneously. During October and November, 1954, all stock down to and including four month old calves were vaccinated. Approximately 1,200 steers and work oxen were inoculated with UM vaccine and 1,400 heifers, breeding stock and bulls with OH vaccine. Thereafter, all calves were vaccinated at four months and

again at weaning. Post-vaccinal swellings appeared at the site of inoculation, but gradually disappeared. The cattle were re-vaccinated in September, 1955, with UM vaccine. To date, that is 19 months since the first vaccination, no deaths from blackquarter have occurred. The apparent success did not allow differentiation between UM and OH vaccines.

TABLE 3

POTENCY OF VACCINES

Prepared from bacterial cells and culture filtrate, tested in sheep.

Type of vaccine	*Vaccinated*	*Controls*
Culture filtrate	3/3	8/8
Bacterial bodies	0/4	
"Normal"	0/4	

Numerator = Deaths
Denominator = Number of sheep challenged.

Duration of Immunity Experiments

Presumed Natural Challenge

The following observations, which are of interest, were made during the field trials. Group 1. At the beginning of May, 1955, 20 heifers and 20 steers, vaccinated in October, 1954, were placed in a paddock which was stated by the owner to be heavily infected with blackquarter. At the end of May, 1956, all remained healthy. Group 2. Twenty bulls and 20 heifer calves first vaccinated in April, 1955 and again in August, 1955, were still healthy at the end of May, 1956. Group 3. One hundred steers and 100 cows vaccinated in October, 1954, were disposed of during the period December, 1955, and May, 1956. Although none was revaccinated all remained healthy until sold.

Parenteral Challenge

Cattle exposed to presumed natural infection. Twenty cattle vaccinated in October, 1954 and kept on the farm used in the field trials, were challenged intramuscularly with 0·1 ml. of a virulent *Cl. chauvoei* culture together with calcium chloride 18 months after vaccination. All survived challenge although one showed a severe reaction at the site of injection. The six unvaccinated sheep controls died.

Cattle kept on a presumed blackquarter-free farm. The results of two experiments are recorded in Table 4. Cattle were challenged 7, 9, 14 and 15 months after vaccination with 0·1 ml. of *Cl. chauvoei* culture with calcium chloride added and all survived. Two out of three of those challenged ten months after vaccination died. All the unvaccinated control sheep died.

TABLE 4

DURATION OF IMMUNITY

Experiments on cattle kept on a presumed blackquarter free farm

Experiment number	*Period since last vaccination (months)*	*Vaccinated*	*Controls*
1	7	0/3	6/6
	9	0/3	
	10	2/3	
2	14	0/2	2/2
	15	0/2	

Numerator = Deaths
Denominator = Number of cattle challenged.

DISCUSSION

The preparation of blackquarter vaccine at Kabete by the old method was laborious and costly. The production method described above overcame these disadvantages and laboratory trials indicated that the vaccine produced was efficient in protecting both sheep and cattle against parenteral challenge with *Cl. chauvoei*. Field trials are notoriously misleading in the absence of adequate controls. Nevertheless, the expected annual death rate in our trials was 14 ± 6, whereas, in fact, none of the vaccinated cattle contracted blackquarter. The duration of immunity test in cattle kept in an area believed to be heavily infected showed that cattle were immune to challenge 18 months after vaccination. Cattle kept for 15 months in an area believed to be free of the disease were also immune, but some died on challenge 10 months after vaccination. We assume that the batch of vaccine used was below standard, even though it passed our potency test. Ideally, these tests should be carried out on the type of animal for which the vaccine is prepared, but this is seldom possible.

Our findings on the efficacy of bacterial cells and whole culture of *Cl. chauvoei* as compared with culture filtrate are in accord with other workers (McEwen, 1926; Henderson, 1932; Roberts, 1933; Mason and Scheuber, 1936; Scheuber, 1944; Rodrigues, 1945) and suggest that the medium of choice is that which produces the most luxuriant growth, namely in our series, GCPW.

We cannot support the statement by Rodrigues (1945) that highly pathogenic strains of *Cl. chauvoei* whose virulence has been recently increased by passage through guinea pigs, are necessary for obtaining potent antigens as the vaccine strain used in these investigations was relatively avirulent. Five ml. of culture failed to

kill sheep and 1·0 ml. failed to kill guinea pigs when inoculated intramuscularly.

The difficulty encountered when using native sheep in blackquarter experiments, due to varying individual susceptibility, was pointed out by Lindley (1955). He used a flocculation test to eliminate naturally immune Nigerian Dwarf-haired sheep for his experiments, but the test did not prove satisfactory in our hands. Instead, we have relied on preliminary tests prior to challenge, both to verify the virulence of the challenge culture and the susceptibility of the batch and type of sheep used.

CONCLUSIONS

A method for the large scale production of liquid blackquarter (*Cl. chauvoei*) vaccine is described. The medium used was four per cent peptone water containing 0·5 per cent glucose and 0·05 per cent cysteine hydrochloride.

The vaccine was fully antigenic after storage at room temperature (18 to 23°C.) for 19 months.

Cattle kept on an infected farm withstood challenge 18 months after vaccination. The duration of immunity induced in cattle kept in a blackquarter-free area, was at least nine months and probably longer.

ACKNOWLEDGMENTS

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17-HYDROXYCORTICOSTEROIDS AND LEUCOCYTES IN THE BLOOD OF DAIRY CATTLE

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INTRODUCTION

Shaw (1947), believing that bovine ketosis was similar in some respects to an adrenal cortical insufficiency, attempted the treatment of four cases of ketosis with an adrenal cortical extract and found a marked improvement in the condition of the animals. Subsequently Shaw, Hatziolos and Leffel (1950) found that cortisone was beneficial in the treatment of ketosis and observed that in ketotic cows the adrenal cortices and anterior pituitary showed evidence of response to stress. On the basis of such evidence they put forward the theory that bovine ketosis resulted from adrenal cortical insufficiency following stress reactions. Their evidence in support of this view is somewhat indirect, being concerned largely with "replacement therapy" (the treatment of ketosis with cortisone, corticotrophin and similar hormones) and the subnormal response, as judged by increase in blood glucose and decrease in circulating eosinophils, of ketotic cows to injections of corticotrophin or adrenaline (Shaw, Hatziolos, Leffel, Gill and Chung, 1952; Shaw, Brown, Gessert and Chung, 1954). Puntriano (1951) has put forward a similar theory of the aetiology of ketosis, and has found that the urinary excretion of glucocorticoids by ketotic cows is less than that of normal cows (Puntriano, 1952).

The ketotic cow shows marked deviations from normality in blood composition, in particular showing marked hypoglycaemia and ketonaemia. If one accepts the findings of Shaw and of Puntriano, there should also be evidence of decreased adrenal cortical secretion of hormones, for example a decreased level of blood adrenal cortical steroids. The present work was undertaken in the hope that observations on the sequence of these changes in blood composition might prove helpful in assessing certain of the current views on the aetiology of bovine ketosis. When it was begun, no reports had been published of estimations of adrenal steroids in the blood of dairy cows, but recently Robertson and Mixner (1954, 1956) have described the estimation of plasma 17-hydroxycorticosteroids in cattle. Since it was desirable that the observations should extend for some time before the appearance of clinical symptoms, it was decided to use as subjects cows with a history of ketosis in previous lactations, in the hope that some of these would develop ketosis in the lactation under study. Unfortunately, none of the animals studied developed ketosis, and so the findings reported here relate entirely to clinically normal

animals. The present paper concerns observations on leucocytes and on plasma 17-hydroxycorticosteroids: observations on blood glucose, ketones and volatile fatty acids will be given in a subsequent paper.

METHODS

Estimation of Plasma 17-Hydroxycorticosteroids

The method used was that of Bayliss and Steinbeck (1953) with certain modifications. It was found that emulsification during extraction of the plasma with chloroform could be avoided if the volume of chloroform used for extraction was made at least twice as great as the volume of plasma. With this greater proportion of chloroform, the extraction of 17-hydroxycorticosterone added to plasma was complete with two extractions. It was found that unless chloroform extracts of bovine plasma were washed carefully with dilute sodium hydroxide the final methanol-sulphuric acid mixtures were turbid and quite unsuited to spectrophotometry. In the chromatography of plasma extracts a mixture of magnesium trisilicate and 'Celite' was used as adsorbent, as described by Nelson and Samuels (1952).

Procedure. Oxalated plasma, 60 ml., was extracted twice with chloroform, 150 ml., the mixtures being shaken for 1 min. in each extraction. The combined chloroform extracts were washed once with 0·1 N sodium hydroxide, 20 ml., and twice with water, 20 ml., each washing being back extracted with an equal volume of chloroform. The washed extract was dried over anhydrous sodium sulphate, filtered and evaporated almost to dryness at 40 to 45°C. in vacuo.

Magnesium silicate—Celite mixture (1:1 w/w), 1·5 g., was suspended in chloroform in a chromatogram tube fitted with a sintered glass disc (1 cm. dia., porosity 3) and the column packed under pressure, 5 lb. N_2/sq. in. The plasma extract was applied to the column in chloroform, 5 ml., washed on with two 2 ml. portions of chloroform, and the chromatogram developed with chloroform, 25 ml., 2 per cent (v/v) ethanol in chloroform, 25 ml., and 20 per cent (v/v) ethanol in chloroform, 25 ml. The third eluate was collected and evaporated to dryness at 40 to 45°C. in vacuo.

The residue from this eluate was dissolved in methanol, 2·5 ml., and two 1·0 ml. portions taken. To one was added 62 per cent (v/v) sulphuric acid, 2·0 ml., and to the other 0·16 per cent (w/v) phenylhydrazine hydrochloride in 62 per cent (v/v) sulphuric acid. Reagent blanks and chromatogram blanks (from a column with no plasma extract added) were also prepared. The mixtures were heated at 60°C. for 30 min., and after cooling the optical densities were read at 410 mμ in 1 cm. cells in a Unicam spectrophotometer. The calculation of results was as described by Bayliss and Steinbeck (1953). Using 17-hydroxycorticosterone as standard, the regression equation of the colour reaction was found to be: weight of 17-hydroxycorticosterone (μg.) = 0·06 + 58·9 × optical density. In twelve experiments the recovery of 17-hydroxycorticosterone added to plasma ranged from 73 per cent to 90 per cent, the mean recovery being 78·5 per cent.

Leucocyte determination. Total leucocytes were determined as described by Boddie (1950) and differential counts were made as described by Coffin (1953).

Collection of blood samples. Blood was collected from the jugular vein (13 b.w.g. needle) directly into wide mouth bottles containing ammonium oxalate-potassium oxalate mixture (3:2 w/w). All blood samples were collected at the same time of day, prior to afternoon milking, and all analyses were begun within about 45 min. of the collection of the samples.

RESULTS

The animals studied were Ayrshire cows on farms near the University of Liverpool Veterinary Field Station. Six cows with a history of ketosis in their 1954/1955 lactations were selected, all being due to calve between November 1955 and April 1956. Sixteen cows with no previous history of ketosis were also available, ten of these being due to calve in the same period as the cows with a history of ketosis. These normal animals were selected so that each cow with previous ketosis was studied with two or three normal cows on the same farm, all being due to calve within a few days of each other. The animals were bled at approximately weekly intervals from ten weeks before the expected date of calving to ten weeks after calving, or for part of that period.

Of the six cows with a history of ketosis, one aborted twin foetuses one month before the expected date of calving, and the results from this cow have been excluded from the consideration of results. None of the remaining five cows with a history of ketosis developed ketosis in the lactation under study. Since all the animals studied were clinically normal and healthy it seemed unlikely that any differences would be found between normal cows and cows with a history of ketosis.

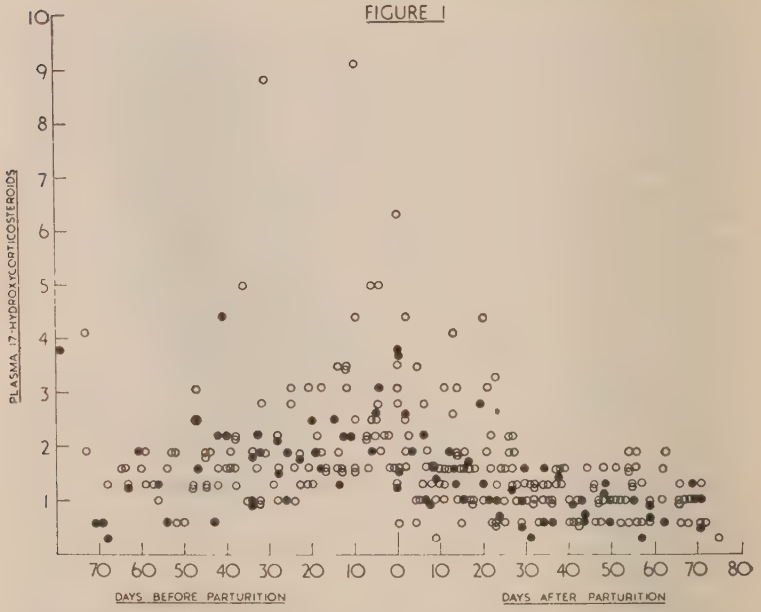
Plasma 17-hydroxycorticosteroids

Comparison of normal cows and cows with previous ketosis. The levels of plasma 17-hydroxycorticosteroids for 16 normal cows (223 results) and 5 cows with a previous history of ketosis (81 results) are shown in Fig. 1, from which it may be seen that most of the results are below 5 μg. 17-hydroxycorticosterone per 100 ml. plasma. The mean and standard deviation for normal cows prepartum were $2{\cdot}2 \pm 1{\cdot}4$ μg. 17-hydroxycorticosterone/100 ml. plasma, and for cows with previous ketosis $1{\cdot}9 \pm 1{\cdot}0$ μg. per cent. For the difference between these means 't' was 1·069 for 124 degrees of freedom, P ca. 0·3. The mean and standard deviation for normal cows postpartum were $1{\cdot}4 \pm 0{\cdot}8$ μg. 17-hydroxycorticosterone/100 ml. plasma, and for cows with previous ketosis $1{\cdot}2 \pm 0{\cdot}5$ μg. per cent. For the difference between these means 't' was 2·067 for 176 degrees of freedom, $P > 0{\cdot}2$. It is, therefore, apparent that no marked difference exists between the two groups of animals.

The results from normal animals showed an apparent seasonal variation, and on analysis this variation was found to be significant. Some of the variation seen from month to month was undoubtedly due to fluctuations in the numbers of calvings per month. When the

results are arranged so that the frequency of calvings is more uniform the seasonal variation is still found (Table 1). In the case of both prepartum and postpartum results, the values found in the summer months are significantly higher than those found in the winter months.

FIGURE 1



Plasma levels of 17-hydroxycorticosteroids (expressed as μg. 17-hydroxy-corticosterone/100 ml. plasma) of cows with previous ketosis (●) and of normal cows (o) before and after parturition.

For normal animals there is a highly significant difference between the postpartum results for the period July to September and those for the period October to December, 't' being 4·06 for 71 degrees of freedom, $P<0{\cdot}001$. No such difference exists between the postpartum results for other periods. Similar findings are obtained from the prepartum results. Thus for the normal animals there is a highly significant difference between the results for the period June to October and those for the period November to December, 't' being 4·27 for 87 degrees of freedom, $P<0{\cdot}001$. While there is a difference in the numbers of calvings in these two periods, the monthly means for the period June to October are uniformly high, and it seems unlikely that differences in the numbers of calvings contribute to the observed difference. In the case of the slight differences between the results for animals with previous ketosis for the two periods tabulated, it seems possible that here

variation in the numbers of calvings is a contributory factor, particularly in the case of the prepartum results. When the results from normal animals obtained during the same periods as the results from animals with previous ketosis are compared, no significant difference is found (Table 1). For subsequent consideration it will be assumed that no difference exists between the results from normal animals and the results from animals with previous ketosis.

TABLE 1

SEASONAL VARIATION IN PLASMA 17-HYDROXYCORTICOSTEROIDS

Comparison of normal cows and cows with previous ketosis.
(Results expressed as μg. 17-hydroxycorticosterone/100 ml. plasma).

	Prepartum results					
Period	*Normal cows*			*Cows with previous ketosis*		
	No. of calvings	*No. of results*	*Mean±S.D.*	*No. of calvings*	*No. of results*	*Mean±S.D.*
June-October ..	8	42	2·9±1·9			
November-December	2	15	1·6±0·5	0	17	1·7±1·0
January-March ..	6	32	1·7±0·5	5	19	2·2±1·0
November-March ..	8	47	1·7±0·5	5	36	1·9±1·0

Comparison of means, November-March: 't'=1·234 (81 degrees of freedom) P>0·2

	Postpartum results					
July-September ..	5	30	2·1±1·1			
October-December ..	5	43	1·3±0·4			
January-March ..	5	38	1·1±0·6	4	37	1·1±0·4
April-May	1	22	1·2±0·5	1	8	1·5±0·7
January-May ..	6	60	1·1±0·6	5	45	1·1±0·5

Comparison of means, January-May: 't'=0·183 (103 degrees of freedom), P>0·8

Changes in plasma 17-hydroxycorticosteroids before and after parturition. The mean values and standard deviations of plasma 17-hydroxycorticosteroids for all animals for 10 day periods over the period studied are shown in Table 2. There is an obvious tendency for the level of plasma 17-hydroxycorticosteroids to increase over the prepartum period, although the increase is most marked in the 10 days immediately prior to parturition. The mean for this period is significantly higher than the mean for the preceding 10 day period. There is a marked decrease in plasma 17-hydroxycorticosteroids at parturition, and thereafter there is a gradual decline until about 30 days postpartum, after which no further change

TABLE 2

SIGNIFICANCE OF CHANGES IN PLASMA 17-HYDROXYCORTICOSTEROIDS BEFORE AND AFTER PARTURITION

(Results expressed as μg. 17-hydroxycorticosterone/100 ml. plasma)

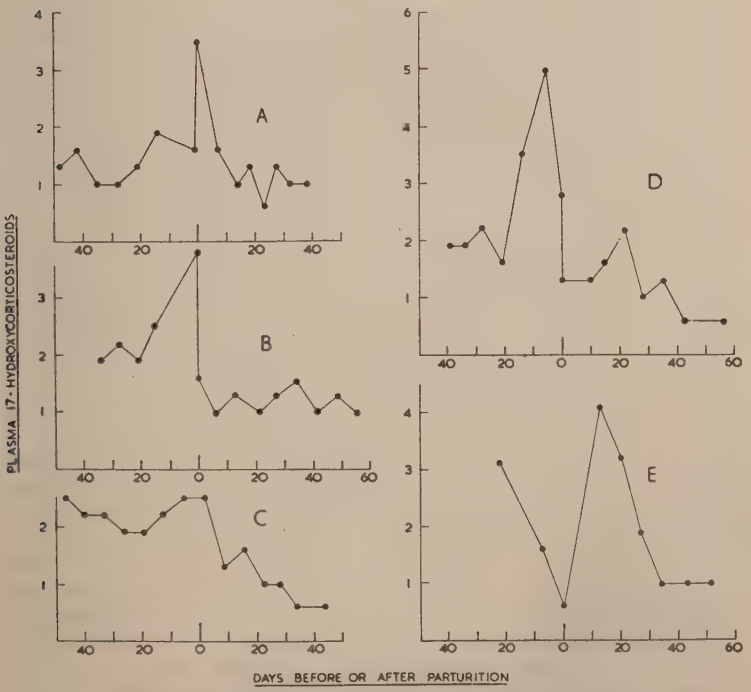
	Days before parturition							*Days after parturition*						
	>60	*60-51*	*50-41*	*40-31*	*30-21*	*20-11*	*10-0*	*0-10*	*11-20*	*21-30*	*31-40*	*41-50*	*51-60*	*>60*
Mean	1·7	1·4	1·8	2·2	1·9	2·2	3·0	1·8	1·8	1·5	1·1	1·0	1·0	1·0
Standard deviation ..	1·2	0·5	0·9	1·7	0·7	0·7	1·7	0·9	0·9	0·8	0·4	0·4	0·5	0·4
No. of results	12	11	16	22	19	20	26	32	28	32	24	22	20	20
Degrees of freedom ..	21	25	36	39	37	44	56	58	58	54	44	40	38	
't'	0·87	1·42	0·76	0·69	1·26	2·09	3·56	0·42	1·39	2·10	1·14	0·49	0·33	
P·	0·4	0·1	0·4	0·5	0·2	0·02	0·001	0·9	0·1	0·02	0·2	0·6	0·7	

The comparison of means are of the two consecutive means above the comparison figures.

occurs. These tendencies in the results are seen equally clearly in Fig. 1.

Considerable variation was seen however in the changes in plasma 17-hydroxycorticosteroids of individual animals as illustrated in examples A to E (Fig. 2). While some cows showed increasing plasma 17-hydroxycorticosteroids as pregnancy progressed, both the rate and extent were variable. In some the increase was reasonably progressive, as in B, whereas in others the greatest increase was seen immediately prior to parturition, as in A where there was a twofold increase in the 24 hr. period prior to parturition. About two-thirds of the animals showed increasing plasma 17-hydroxycorticosteroids prepartum, while most of the remainder showed more or less uniformly high levels over the prepartum period studied, as in C. Four cows showed a decrease in plasma 17-hydroxycorticosteroids prepartum, as in D and E. In these cases the decreases occurred within two weeks of parturition, and followed a general increase.

FIGURE 2



Examples of the plasma levels of 17-hydroxycorticosteroids (expressed as μg. 17-hydroxycorticosterone/100 ml. plasma) of individual cows, showing the various changes in level which may occur before and after parturition.

In almost all cases there was a sharp decrease postpartum, the decrease being usually very rapid. In those cases bled within 12 hours before parturition and within 12 hours after parturition, most showed a marked decrease in the 24 hour period. After the initial postpartum decrease some cows showed reasonably steady levels as in B or generally decreasing levels, as in C. About two thirds of the animals showed such postpartum changes; the remaining third, however, showed a subsequent temporary increase postpartum. These peaks occurred in the period 2 to 4 weeks postpartum.

While about two-thirds of the individual animals studied showed prepartum and postpartum changes essentially similar to the mean picture, some cows showed deviations, notably decreases prepartum and peaks postpartum.

Changes in Leucocytes before and after Parturition

For most of the blood samples in which plasma 17-hydroxycorticosteroids were determined, total and differential leucocyte counts were also estimated.

About three-quarters of the animals showed prepartum increases in total leucocytes, the greatest being observed in the two weeks before parturition. The remaining animals showed no marked change in total leucocytes before parturition, but as none of these was observed at less than five days prepartum, they too may have changed in the immediate prepartum period. The marked increase seen at about 10 days prepartum is significant, 't' $=1{\cdot}82$ for 41 degrees of freedom, $P>0{\cdot}05$. In about 20 per cent of cases the prepartum increase in total leucocytes extended to a day or two after parturition, but all showed a marked decrease in the immediate postpartum period, which was significant, 't' $=2{\cdot}19$ for 52 degrees of freedom, $P>0{\cdot}02$. While the mean postpartum decrease is followed by an increase to the very high prepartum level, and by a subsequent decrease to a relatively constant level, there was some variation shown by individual animals. In some, the postpartum decrease was so profound and of sufficient duration as to constitute a very marked depression in total leucocytes, whilst in others the decrease was not very marked. Similarly in the subsequent increase, some animals showed such a marked increase that there was a well defined peak prior to the attaining of a constant level, whilst in others there was no marked peak. The mean values, given in Table 3, are a reasonably good representation of the levels of most individual animals, the notable variations being the continuance of the prepartum increase for a short time postpartum, and the extent of the postpartum decrease and subsequent increase.

Changes in neutrophils were in essential very similar to the fluctuations in total leucocytes, and in most animals the more prominent changes in total leucocytes could be accounted for by the neutrophils, although in the postpartum decrease in total leucocytes a transient lymphopenia was contributory in some cases.

TABLE 3

LEVELS OF LEUCOCYTES BEFORE AND AFTER PARTURITION

	Days before parturition							*Days after parturition*						
	60	*60-51*	*50-41*	*40-31*	*30-21*	*20-11*	*10-0*	*0-10*	*11-20*	*21-30*	*31-40*	*41-50*	*51-60*	*60*
Total leucocytes														
Mean..	12760	13260	14260	13910	12960	13260	14810	13040	14940	13780	14100	13270	13130	14360
±S.D.	3360	2370	3720	2610	2450	2720	2700	3000	3930	3270	2480	2360	1550	3030
Lymphocytes														
Mean..	6980	7340	7900	7210	6990	6590	6670	6320	6730	6850	6780	6220	6980	7080
±S.D.	1860	1380	1910	1860	1690	1870	1510	1500	1850	1720	1400	1190	1080	2110
Neutrophils														
Mean..	3250	3520	3770	4290	3860	4670	6010	5000	6080	4670	4780	4480	3980	4730
±S.D.	980	650	1340	1040	710	1260	1470	2430	2320	1590	1530	1840	1160	1390
Eosinophils														
Mean..	1320	980	1200	1060	960	730	700	470	810	920	1190	1200	1010	1200
±S.D.	600	620	730	530	600	510	500	460	490	730	800	1000	600	700
No. of results	12	10	15	21	18	21	23	31	27	27	23	23	17	17

Most animals showed a tendency to increasing neutrophils prepartum, although in some cases the change was noticeable only in the two weeks before parturition, and in some the increase extended to a day or two after parturition. In all cases there was a marked decrease postpartum, and as in total leucocytes, some animals showed a very profound decrease before attaining a constant postpartum level, while others showed a marked peak after the postpartum decrease. For the difference between the means for 20-11 days and 10-0 days prepartum 't' = 3·05 for 41 degrees of freedom, $P > 0.001$. For the decrease at parturition 't' = 1·74 for 52 degrees of freedom, $P > 0.05$. The changes at 10 days and 20 days postpartum are also significant at 5 per cent and 1 per cent respectively.

There were no marked changes in lymphocytes. Most animals showed a very slight lymphopenia in the immediate prepartum period, although in some cases the lymphopenia was more noticeable in the immediate postpartum period.

Most animals showed no marked change in eosinophils until the immediate prepartum period, when there was an eosinopenia. In some the decrease in eosinophils continued into the postpartum period, and in some the greatest decrease in eosinophils was seen in the period immediately after parturition. While for the mean the decrease prepartum is significant, the most significant change is the decrease at parturition; 't' = 2·75 for 52 degrees of freedom, $P > 0.001$. The correlation between plasma 17-hydroxycorticosteroids and eosinophils was calculated for each animal, and of the 21 values of correlation coefficient so obtained 14 were negative, ranging from $r = -0.104$ to $r = -0.730$, while the remaining seven values were positive, ranging from $r = +0.113$ to $r = +0.379$. A pooled estimate of the correlation coefficient was obtained using Fisher's z transformation and was found to be $r = -0.291$. Thus only in a few cases was there good correlation between plasma 17-hydroxycorticosteroids and eosinophil levels, and from the pooled estimate correlation was rather poor.

DISCUSSION

From this work it would appear that the level of 17-hydroxycorticosteroids in the peripheral blood of cattle is quite low, and considerably lower than the level in man. Bayliss and Steinbeck (1953) found a range of 5·0 to 20·4 μg. per cent for normal non-pregnant women, and Bayliss, Browne, Round and Steinbeck (1955) found values of 11 to 42 μg. per cent for the last three months of pregnancy in women, and values of 3 to 42 μg. per cent for the early postpartum period. In the present work the values for the last 2 to 3 months of pregnancy in cows ranged from 0·3 to 9·1 μg. per cent, and postpartum from 0·3 to 4·5 μg. per cent. These values are lower than the values found by Robertson and Mixner (1956) who

report values of 2·14 to 8·40 postpartum in cows, and values of 6·90 to 17·62 for the last six weeks of pregnancy. Forsius and Haikonen (1955), using the method of Bayliss and Steinbeck (1953) found values of 0 to 4·5 μg. per cent in sheep.

Robertson and Mixner (1956) attempted to apply to bovine plasma the method of Nelson and Samuels (1952) but found that the measurement of absorption at 410 mμ of the steroid phenylhydrazone was impaired by excessive chromogenesis by the sulphuric acid of the reagent. To avoid this they adopted ethyl acetate in place of chloroform for the extraction of plasma, and omitted the chromatography of the plasma extract. While in the present work it was found that the application to bovine plasma of the method of Bayliss and Steinbeck (1953), which is an improved modification of the method of Nelson and Samuels (1952), gave final reagent mixtures unsuited to spectrophotometry, this was believed to be due to turbidity which could be avoided by washing the plasma extract with dilute sodium hydroxide. No comparison has yet been made of the present method with that of Robertson and Mixner (1956) and therefore no reason can be given for the discrepancy in results.

A considerable number of steroids are present in adrenal cortical tissue, but it now seems clear that only a few are found in adrenal venous blood, and so presumably in peripheral blood. The adrenal cortical steroids of biological importance have certain structural features in common, namely an α ketol side chain and a $\triangle_4$ 3-ketone. Hechter, Zaffaroni, Jacobsen, Levy, Jeanloz, Schenker and Pincus (1951) isolated a number of α ketolic steroids from blood perfused through bovine adrenals. Corticosterone and 17-hydroxycorticosterone were present in considerable amounts, and cortisone and 11-dehydrocorticosterone were present in smaller quantities, as were a number of unidentified substances, possibly reduction products of these other steroids. Bush (1953) in a study of adrenal venous blood, found in all the species studied that corticosterone and/or 17-hydroxycorticosterone constituted some 85 to 100 per cent of the $\triangle_4$ 3-keto steroid detected. In bovine adrenal venous blood corticosterone and 17-hydroxycorticosterone were present in approximately equal amounts. It would therefore seem probable that in bovine peripheral blood the main biologically active component is 17-hydroxycorticosterone, and consequently estimation of blood 17-hydroxycorticosteroids should provide a reliable index of adrenal cortical function.

Blood 17-hydroxycorticosteroids can be estimated by the methods dealt with at the beginning of this discussion. It seems probable that the 17-hydroxycorticosteroids of blood are present in the plasma only (Morris, 1953), and these methods use plasma. The methods have in common their colour reaction (Porter and Silber, 1950), which is based on the reaction between the steroid and phenylhydrazine in strong sulphuric acid, and which is claimed to

be specific for α ketolic steroids with a hydroxyl group at C17. From the work of Bush (1953) the only such steroid present in bovine blood in appreciable amount should be 17-hydroxycorticosterone, although there may also be present metabolic products of 17-hydroxycorticosterone. Bayliss and Steinbeck (1953) in an attempt to verify the specificity of their method, found that about half of the 17-hydroxycorticosteroid estimated was 17-hydroxycorticosterone, the remainder possibly being reduction products of 17-hydroxycorticosterone or of cortisone. If similar substances are present also in bovine peripheral blood, their inclusion in the material estimated does not detract from the value or specificity of the method.

The results presented in this paper show clearly an increased adrenal cortical activity in pregnancy in dairy cows. It is difficult to choose from these results a value of plasma 17-hydroxycorticosteroid for a "normal cow", but if the relatively constant value of about 1·0 μg. per cent at 5 to 10 weeks postpartum is taken as normal, then increased levels were observed throughout the last ten or eleven weeks of pregnancy. This work has not determined at what stage of pregnancy the increase in plasma 17-hydroxycorticosteroid begins. Bayliss, Browne, Round and Steinbeck (1955) found that in women the increase in adrenal cortical activity began at about the third month of pregnancy, and also found some variation in the changes shown by individuals. Somewhat similar variation was noted among individual cows in the present work.

Although most cows showed a decrease in adrenal cortical activity at about parturition, too few were examined less than 24 hours prepartum and immediately postpartum to decide how closely the decrease in plasma 17-hydroxycorticosteroids was associated with parturition. The changes in the first few weeks after parturition are of interest, particularly the peak levels of plasma 17-hydroxycorticosteroid shown 2 to 3 weeks postpartum by about one third of the cows. This peak appeared to be associated with maximum lactation, but it does not necessarily follow that it indicates a response to a 'lactation stress'. In fact the most marked increase in steroid was observed in the cow with the lowest milk yield (about 25 lb. per day) and with almost constant milk yield for the first five weeks postpartum, while cows giving milk yields of about 60 lb. per day and showing a very marked peak in milk yield showed little or no increase in plasma 17-hydroxycorticosteroid.

The finding of an apparent seasonal variation in plasma 17-hydroxycorticosteroid was made too late for any attempt to be made to determine the reason for the variation. That the highest levels were found in the summer months, and that quite a sharp change was seen about October might suggest temperature as a contributory factor, particularly since the summer of 1955 was on the whole rather warm and dry. An alternative explanation might be that in summer months the animals were more active, and in fact were bled

soon after they had walked from the pasture to the dairy. In the winter months they were of course indoors and thus were not exercised just before bleeding.

The changes in leucocytes at parturition in the normal animal are well known, and it has been shown that similar changes in leucocytes may be elicited in the non-parturient animal by the administration of corticotrophin (Smith and Niedermeier, 1953; Merrill and Smith, 1954). It was, therefore, to be expected in the present work that where increases in plasma 17-hydroxycorticosteroids were observed these would be associated with changes in leucocytes. The association observed in individual animals varied considerably, an obvious example of this being the positive correlation between plasma 17-hydroxycorticosteroids and eosinophils shown by 7 cows. When it is borne in mind that the bleedings were done at weekly intervals it is possibly not surprising that some variation was found, and it might be expected that analysis at more frequent intervals would show better correlation between plasma 17-hydroxycorticosteroids and leucocytes.

From the results given in this paper it would appear that any attempt at assessing adrenal function on the basis of say one or two counts of eosinophils might be subject to considerable error. There is such a wide variation in individuals in leucocyte levels that there could be no question of assessing adrenal cortical function other than by frequent counts over a period of time. Changes then observed in leucocytes might form a reasonably reliable qualitative index of change in adrenal cortical function, but such observations would have no quantitative significance. The use of the eosinopenic response to adrenaline or corticotrophin is becoming commonly used as a means of assessing adrenal cortical function. Shaw *et al.* (1952), used such tests in ketotic cows and found that in the ketotic cow the adrenal cortical response to adrenaline and to corticotrophin was subnormal. No attempt has yet been made to determine the reliability of such tests in dairy cows, but Hunter, Bayliss and Steinbeck (1955) found in man that while adrenaline invariably produced a marked eosinopenia, it was difficult to relate such changes to changes in plasma 17-hydroxycorticosteroids estimated simultaneously.

The only estimations of plasma 17-hydroxycorticosteroids in ketotic cows so far published were by Robertson, Lennon and Mixner (1955) who found that in ketotic cows the plasma 17-hydroxycorticosteroids were slightly higher than normal. This finding is not in accord with Shaw's concept that ketosis results from adrenal cortical failure. It is intended to pursue the original objective of this present work, which is to determine how adrenal cortical function in the ketotic cow differs from normal, and to determine the sequence of changes in adrenal cortical and other metabolic functions.

CONCLUSIONS

Plasma 17-hydroxycorticosteroids were determined and leucocyte counts were made in 22 dairy cows for some time before and after parturition. The mean values of plasma 17-hydroxycorticosteroids were higher prepartum than postpartum. The highest values were found 1 to 2 weeks prepartum and there were marked decreases at parturition.

Total leucocytes and neutrophils showed significant increases 1 to 2 weeks prepartum, with sharp decreases at about parturition. There was a significant eosinopenia 1 to 2 weeks prepartum. Changes in lymphocytes were not significant.

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THE DETECTION OF *M. JOHNEI* IN SECTIONS BY FLUORESCENT EXAMINATION

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INTRODUCTION

During the course of the histological examination of the tissues of goats infected with *M. johnei* (Harding, 1957) it soon became apparent that the routine Ziehl-Neelsen staining technique was not satisfactory for the demonstration of small numbers of organisms in sections, as cultures made from the organs showed the presence of *M. johnei* in many cases where they could not be demonstrated histologically. The difference was not so marked in organs such as the intestine and mesenteric lymph nodes where lesions tended to be prominent and organisms numerous, and was greatest in the spleen where the absence of clear-cut lesions did not permit the careful searching of areas previously selected with the lower powers of the microscope.

Gray (1953) had similar difficulties in detecting small numbers of mycobacteria in experimental infections, and found that by using a fluorescent technique the detection of organisms was greatly improved. Fluorescence for the demonstration of acid-fast bacilli was first described by Hagemann (1938), but the method demanded special equipment for using ultra-violet radiation. Graham (1942) showed that light at the blue end of the visible spectrum could excite fluorescence, and described a technique in which a tungsten filament projection lamp was the source of light and an ammoniacal solution of copper sulphate was used as a filter to remove the unwanted higher wavelengths. A deep yellow filter in the microscope absorbed all the light transmitted by the copper sulphate, so that the field appeared black and the only light reaching the eye came from fluorescent material in the preparation being examined. This simplified optical arrangement was subsequently modified by Matthaei (1950). Gray (1953) utilised Matthaei's system, but improved the staining methods previously employed by adding rhodamine to the carbol-auramine fluorescent stain, and by drying the sections from water instead of dehydrating through the alcohols in the usual way.

METHODS

The staining technique differed only in minor details from the method laid down by Gray (1953). Sections were taken down to water, paying particular attention to the removal of all traces of wax and any mercury deposit, placed on a hot plate at 70°C. and flooded with the carbol-auramine-rhodamine stain. After 10 minutes the sections were washed carefully in tap water, and decolourised using three changes of one per

cent. acid-alcohol in five minutes. After a further careful wash the sections were placed on the hot plate until thoroughly dry and then mounted in liquid paraffin. Care was necessary to avoid dust settling on the section while it was drying, as many particles were found to be fluorescent, although usually of a different colour from acid-fast material in the section.

The Baker 'Projectolux' was found to be a suitable apparatus for examining the sections, as it has a high light output and there is a space below the sub-stage condenser which will accommodate the necessary depth of copper sulphate solution. The sub-stage condenser supplied with the instrument was replaced with an ordinary Abbé type, as this was more convenient to use with liquid paraffin between it and the slide, which is necessary to attain a sufficiently high numerical aperture. Accurate centering and focussing of the whole optical system was essential. In use, a disc of Kodak G15 gelatin filter was placed in the ocular, a slide stained as above was placed in position on the stage and ammoniacal copper sulphate solution was added to a flat-bottomed vessel placed below the sub-stage condenser until the optimum condition was reached. This was considered to be when the tissue showed as a faint olive green against a dark background. If there was insufficient solution the background was bright; if there was too much the tissue became almost indistinguishable. Organisms which had retained the auramine-rhodamine stain stood out a brilliant golden yellow. Gray (1953) recommended that the microscopy should be carried out in a shaded corner of the room, but it was found that better results were obtained by working in a dark-room.

Sections were examined with the $\frac{2}{3}$rd and $\frac{1}{6}$th objectives, and a $\times 8$ ocular. The whole section was first scanned rapidly using the $\frac{2}{3}$rd, which allowed the orientation of the tissue and also detected any clumps of organisms present. Groups of four or more bacilli could be distinguished at this magnification and occasionally it was possible to see groups of only two or three organisms. The low power findings were always confirmed with the high power lens and, if nothing had been observed with the low power, the section was systematically searched with the high power. When organisms were found there was rarely any doubt as to their identity, though in a very small number of sections fluorescent particles were observed which could not accurately be identified. Where any doubt existed the section was considered to be negative.

Where practicable, serial sections were used in order to make the comparison as accurate as possible. The Ziehl-Neelsen section was examined first and the fluorescent section second. Where the Ziehl-Neelsen section had proved negative at the first examination it was sometimes possible to find acid-fast organisms in it when the area containing them had been indicated by the fluorescent slide, but in these cases the result was nevertheless entered in the table as negative to the Ziehl-Neelsen method.

RESULTS

The organs of 43 goats were examined culturally for the presence of *M. johnei* and histologically by the Ziehl-Neelsen and fluorescent methods. Table 1 gives the results obtained from 15 animals selected as representative of those in which the methods disagreed.

TABLE 1

THE PRESENCE OF *M. JOHNEI* IN THE ORGANS

As shown by culture, Z.N. and F.L.M. examinations of histological sections

Goat No.	*Lungs*			*Liver*			*Kidneys*			*Spleen*			*Intestine*			*Mesenteric L.N.*			*Hepatic L.N.*			*Other L.N.*		
	C	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*	*C*	*ZN*	*FLM*
1648	*	—	+	*	+	+	—	—	—	+	+	+	o	+	+	+	+	+	+	+	+			
1665	+	+	+	+	+	+	—	+	+	—	—	+	o	o	o	+	+	+	+	+	+	+	—	+
1667	—	—	—	+	—	+	+	+	+	+	—	+	o	o	o	+	+	o	+	+	+	+	+	+
1669	+	—	+	—	—	+	+	—	+	+	—	+	+	+	+	+	+	+	+	+	+	+	o	o
1807	+	—	+	—	—	—	+	—	+	+	—	+	o	+	+	+	+	+	—	—	—			
1809	—	—	—	+	+	+	+	—	+	—	—	—	o	o	o	+	—	—	o	o	o			
1811	o	—	+	+	—	+	—	—	—	—	—	+	—	+	+	+	+	+	—	—	—			
A4419	+	—	+	+	—	+	o	o	o	+	—	+	—	—	+	+	—	+	+	—	+	+	—	+
5	—	—	+	—	+	+	—	—	+	—	—	—	+	+	+	+	+	+	—	+	+			
1960	+	+	+	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	+	+			
1962	—	—	+	—	+	+	—	—	—	—	—	+	+	+	+	+	+	+	+	—	+			
1963	+	—	+	+	+	+	—	—	+	+	—	+	+	+	+	+	+	+	o	+	+			
1964	—	+	+	—	+	+	—	—	—	—	—	—	+	+	+	+	+	+	o	o	o			
1966	+	+	+	+	+	+	+	—	+	+	—	+	+	+	+	+	+	+	+	o	o			
1969	+	+	+	—	—	+	+	—	—	+	—	—	+	—	—	+	+	+	o	o	o			
Totals Positive	9	5	13	9	9	14	7	2	9	9	1	11	8	10	11	15	13	13	8	7	9	4	1	3

C=Cultural results. *ZN*=Z.N. stained section. *FLM*=Fluorescent examination.
* Organisms seen in material prepared for culture, but no growth obtained. + Organisms present. — No organisms present.
o Not examined.

In sections of the intestine and mesenteric and hepatic lymph nodes, in which acid-fast bacilli were usually numerous, all three methods gave very similar results. In the other organs cultural and fluorescent microscopy methods were in reasonably close agreement, fluorescent microscopy, however, showing a higher number of positive results in each case. The Ziehl-Neelsen method was markedly inferior, particularly in organs in which acid-fast bacilli were few in number, or in which the absence of easily recognisable lesions made it difficult to select suitable areas to examine.

A small number of faeces samples were examined for the presence of *M. johnei* using the same staining technique as for sections. Although any *M. johnei* present fluoresced brightly it was found that numerous vegetable fibres and other organisms were also fluorescent, so that the advantage of visual contrast was lost. In addition, the morphological characters of *M. johnei* were less easily distinguished than by the routine Ziehl-Neelsen method.

The agreement between cultural examination and fluorescence is close, though a number of discrepancies occur. These sometimes appear to favour one method, sometimes the other. A possible explanation is the patchy distribution of the organisms in the tissues: thus the part of the organ which was cultured may actually have contained organisms, whereas that which was sectioned may not, and vice versa. Also, the very small volume of tissue examined histologically may lead to spurious negative findings in cases where the number of organisms was low. This was shown to be the case by examining a number of sections from a single block. In several instances some sections were found to contain organisms, whereas others, only a few microns distant, contained none.

The difference between the Ziehl-Neelsen and fluorescent methods was very variable, depending mainly upon the number of organisms actually present in the sections under examination. Where organisms were numerous, as in the mesenteric lymph nodes and intestine, the numbers of positive results were almost identical, but where organisms were present only in small numbers fluorescence proved to be very much more sensitive. Where both methods gave positive results it was usually found that organisms could be detected more rapidly by fluorescence than by Ziehl-Neelsen staining. The advantage of the fluorescent method over the Ziehl-Neelsen technique was probably not quite as marked as it appears from the table as there is little doubt that with more protracted examinations a slightly greater number of positives could have been obtained for the Ziehl-Neelsen method.

DISCUSSION

It has been shown by Hagan and Mansfield (1930), Sahai (1940), Levi (1941), and Francis (1943) that in the lesions produced by injecting *M. johnei* into small laboratory animals acid-fast bacilli could often be demonstrated microscopically although they could

not be cultured. Hagan and Levine (1932) showed that dead *M. johnei* retained their characteristic morphology for several weeks after inoculation. It thus seems probable that in organs such as the lung, liver, spleen and kidney in which *M. johnei* seems to be incapable of multiplying to any appreciable extent in the goat, the histological methods were demonstrating bacilli which had been killed by the body defences.

Soltys (1952) reviewed the position regarding fluorescent microscopy for the detection of *M. tuberculosis*. He quoted a number of workers who found that fluorescence was an improvement over conventional methods, and others who considered that the routine Ziehl-Neelsen method was better. Most of the work was concerned with the examination of films and it is interesting to note that the results obtained varied appreciably according to the nature of the secretion being examined. Soltys (1952) concluded that fluorescence could not be recommended as a routine method. Ehrlich (1953) considered that fluorescence was more sensitive than the Ziehl-Neelsen method, but felt that great care was necessary in order to avoid false positive results owing to the difficulty in distinguishing *M. tuberculosis* from saprophytic acid-fast bacilli.

A limited trial of fluorescent microscopy for the detection of *M. johnei* in faeces indicated that it could not be recommended as a routine diagnostic procedure, though it is probable that the method could be improved by adopting Herrmann's (1938) technique for reducing non-specific fluorescence. This consists of counterstaining the film with 0·1 per cent. potassium permanganate solution followed by Loeffler's methylene blue.

For sections the method described proved highly satisfactory, and it would appear that the staining modifications introduced by Gray (1953), used with an optical system capable of yielding a high intensity of light, is as sensitive as any other technique available for the detection of *M. johnei* in the tissues. Attention to detail was important, but no difficulties were encountered and the method appears to be worth a regular place in research techniques.

CONCLUSIONS

Fluorescent microscopy (F.L.M.) of sections was markedly superior to the Ziehl-Neelsen (Z.N.) method for detecting small numbers of *M. johnei* in the tissues of experimentally infected goats. F.L.M. and Z.N. were in agreement in 62 cases, F.L.M. was positive in 36 cases which were negative by Z.N., and in no case was Z.N. positive and F.L.M. negative.

In most organs F.L.M. gave more positive results than did cultural methods. There was agreement in 53 cases, cultures were positive and F.L.M. negative in four, and cultures were negative and F.L.M. positive in 14.

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EFFECT OF FEEDING 2-AMINO-5-NITROTHIAZOLE* AND 2-ACETYL-AMINO-5-NITROTHIAZOLE† ON REPRODUCTION IN THE FOWL

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INTRODUCTION

Recent research has suggested that continuous treatment of flocks over substantial periods is more effective in controlling the spread of histomoniasis (blackhead) than the treatment of individuals with higher concentrations of a drug for short periods (Horton-Smith and Long, 1956). It is therefore important that information be available on the occurrence of possible side-effects, particularly on reproduction, of drugs used in such treatment.

Enheptin (2-amino-5-nitrothiazole) has been used for some considerable time (Waletzky, Clark and Marsen, 1950; Swales, 1950; Jungherr and Winn, 1950), and though found to be effective in control of the disease, caused a reduction in comb size, regression in spermatogenesis in the male (Hudson and Pino, 1952) and delay in onset of egg production in the female (Pino and Hudson, 1953). Joyner (1953) noted no toxic effect as evidenced by body weight and intake of food when poults were fed Enheptin at 0·05 per cent, but a significant reduction in weight gain occurred at a level of 0·1 per cent for 14 days. Enheptin-A (2-acetyl-amino-5-nitrothiazole), an intermediate product of Enheptin, has proved satisfactory in the control of histomoniasis in turkeys and has been reported to be less toxic (Grumbles, Boney and Turk, 1952; Brander and Wood, 1955). Price and Bottorff (1954) noted a significant decrease in fertility (8·2 per cent) when turkeys were fed the drug for a lengthy period, but did not consider this to be of serious economic importance. Price and Gingher (1956) reported no significant differences in weight gain, egg production, egg weight, fertility, hatchability and saleable chicks in groups of birds fed Enheptin-A at 0·0075 and 0·015 per cent for two weeks, nor in birds fed at 0·015 and 0·03 per cent intermittently three days on and four days off for a total of 12 medicated days.

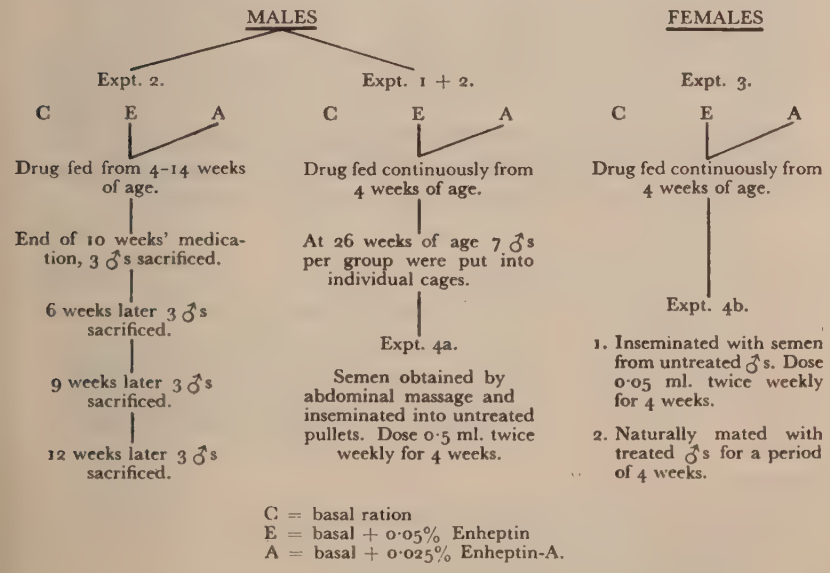
In view of the limited published work, particularly in this country, it seemed desirable to undertake experiments to determine the effect of both drugs on reproduction when fed continuously at the levels recommended in the preventive treatment of blackhead.

* 2-amino-5-nitrothiazole is known in the U.S.A. as Enheptin and in the U.K. as Entramin.

† 2-acetyl-amino-5-nitrothiazole is known in the U.S.A. as Enheptin A and in the U.K. as Entramin A.

EXPERIMENTAL METHODS

Single Comb White Leghorn chicks hatched at this Station were randomized when four weeks of age into six groups of 20 males each, and three groups of 22 females. Brooding and rearing were carried out as described in a previous paper (Cooper and Skulski, 1956). The birds were fed the Station chick starter ration from 0 to 14 weeks and the breeder ration from 14 weeks onward. Enheptin was added to the ration at 0·05 per cent w/w and Enheptin-A at the level of 0·025 per cent w/w in the manner described by Horton-Smith and Long (1956). The birds were individually weighed and the length x width of the comb area measured at frequent intervals (Fig. 1 and 2, Tables 1-3). The protocol of the experiments is detailed in the diagram below.


MALES
FEMALES
Expt. 2.
Expt. 1 + 2.
Expt. 3.
C E A
C E A
C E A
Drug fed from 4-14 weeks of age.
Drug fed continuously from 4 weeks of age.
Drug fed continuously from 4 weeks of age.
End of 10 weeks' medication, 3 ♂s sacrificed.
At 26 weeks of age 7 ♂s per group were put into individual cages.
6 weeks later 3 ♂s sacrificed.
Expt. 4a.
Expt. 4b.
9 weeks later 3 ♂s sacrificed.
Semen obtained by abdominal massage and inseminated into untreated pullets. Dose 0·5 ml. twice weekly for 4 weeks.
1. Inseminated with semen from untreated ♂s. Dose 0·05 ml. twice weekly for 4 weeks.
12 weeks later 3 ♂s sacrificed.
2. Naturally mated with treated ♂s for a period of 4 weeks.
C = basal ration
E = basal + 0·05% Enheptin
A = basal + 0·025% Enheptin-A.

Eggs from individual hens were identified and set once weekly in a forced-draught incubator of the pre-heated air type. Candling individually at 96 hours and tray candling at 18 days was carried out. All eggs appearing clear or containing dead embryos were opened and examined macroscopically for development.

Semen production from males fed the drug continuously from four weeks of age was recorded for a four week period during March 1956 when the birds were 26 to 30 weeks old. Sperm counts on duplicate samples were made weekly using a Fuchs-Rosenthal haemocytometer (Rothschild, 1950).

Testicular tissue was taken immediately after the birds were killed, fixed in 10 per cent formol saline for 24 to 48 hours, embedded in paraffin and sectioned, in some cases serially. Sections were stained routinely with haematoxylin-eosin. Other methods included picro-Mallory for hyaline changes and Putt's carbol-fuchsin for spermatozoa.

RESULTS

Body Weight

The effect of the continuous feeding of Enheptin and Enheptin-A on body weight is shown in Tables 1 and 2. Analysis of variance and the F test shows a highly significant difference in body weight gain between controls and the Enheptin-fed birds of both sexes. Birds fed Enheptin-A showed no such differences. In the groups in which the drug was fed for a 10 week period only, the Enheptin-fed birds gained weight rapidly following discontinuance of the drug and nine weeks later were somewhat heavier than the controls (Table 3). There were no large differences in food consumption, but unless birds are housed in individual cages it is difficult to calculate consumption accurately. It was, therefore, difficult to assess this factor in an estimate of the effect of the drug on body weight, but it is believed that the difference in weight gain was primarily due to the drug and not to the somewhat decreased food intake.

TABLE 1

EFFECT ON BODY WEIGHT OF PULLETS OF THE CONTINUOUS FEEDING OF DRUGS FROM 4 WEEKS OF AGE

Treatment	*Gain in body wt. (grams) after*					
	4 wks.	*8 wks.*	*10 wks.*	*14 wks.*	*18 wks.*	*33 wks.*
Control ..	398·7	743·3	899·0	1145·8	1287·9	1607·0
Enheptin ..	353·7	663·0	791·0	978·4†	1018·8†	1340·5†
Enheptin-A ..	389·1	730·5	882·5	1075·4	1187·7*	1542·5

*Significant P=<0·05
†Highly significant P=<0·01
L.S.D. after 14 weeks drug feeding ·01=101·7 g.
L.S.D. after 18 weeks drug feeding ·01=127·3 g. ·05=95·7 g.
L.S.D. after 33 weeks drug feeding ·01=165·4 g.

Comb Development

A highly significant (P=<·001) effect on comb development occurred in the groups fed Enheptin (Fig. 1). On discontinuing the drug, comb size rapidly increased and nine weeks after cessation of drug feeding the comb area was similar in size to that of the controls (Fig. 2). These findings confirm those of Hudson and Pino (1953). Enheptin-A had no significant effect on comb size, even when fed continuously for 33 weeks.

Egg Production

The controls and Enheptin-A fed groups came into lay at the same time, but the group fed Enheptin were delayed by 14 days. Egg production and the numbers of birds in lay are shown in Table 4. The observation that some of the pullets in the Enheptin-fed groups produced eggs during the experimental period differs from the results of experiments carried out by Pino and Hudson (1953) but it is not known whether this was due to individual variation or to the earlier application of the drug, at 4 weeks of age, with perhaps some adaptation. The combs in these birds were somewhat reduced in size and egg size was also reduced. Examination post-mortem of birds in lay from this group showed no gross abnormalities; the oviduct and ovary were very active. In birds not in lay the oviduct and ovary were quiescent.

TABLE 2

EFFECT ON BODY WEIGHT OF COCKERELS OF THE CONTINUOUS FEEDING OF DRUG FROM 4 WEEKS OF AGE

Treatment	*Gain in body wt. (grams) after*					
	4 wks.	*8 wks.*	*10 wks.*	*14 wks.*	*19 wks.*	*33 wks.*
Control ..	481·3	987·5	1187·4	1516·4	1788·7	1864·2
Enheptin ..	312·0	772·7	945·0	1190·4†	1401·0†	1581·4†
Enheptin-A ..	356·2	936·3	1150·4	1515·3	1710·5	1778·3

†Highly significant P=<0·01
L.S.D. after 14 weeks drug feeding ·01=187·4 g.
L.S.D. after 19 weeks drug feeding ·01=197·6 g.
L.S.D. after 33 weeks drug feeding ·01=248·3 g.

TABLE 3

BODY WEIGHT GAINS OF COCKERELS

Four weeks and over.

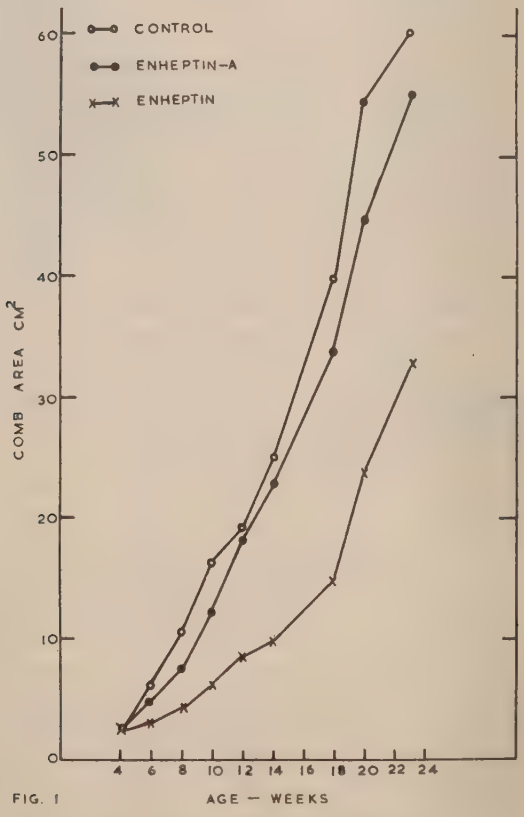
Treatment	*During treatment*			*After treatment*		
	Body wt. gain (grams) after			*Body wt. gain (grams) after*		
	4 wks.	*8 wks.*	*10 wks.*	*4 wks.*	*6 wks.*	*9 wks.*
Control ..	464·8	921·3	1155·9	1449·4	1649·2	1622·0
Enheptin ..	412·2	843·9	1009·4	1451·2	1603·1	1676·4
Enheptin-A ..	481·4	890·8	1123·2	1467·1	1584·6	1699·0

Semen Production

Semen production was recorded for March only, the birds then having been fed the drug for 22 weeks. Collections were made four times weekly using the abdominal massage technique (Burrows and Quinn, 1937; Cooper, 1955). Mean volumes and density were as follows:

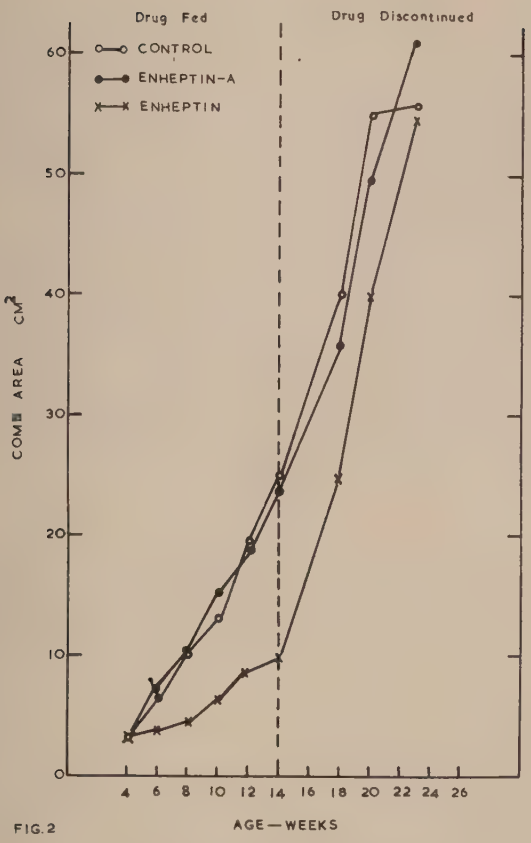
	Vol./collection	*Mean density*
Controls	0·339 ±0·06 ml.	$4{\cdot}35 \times 10^9$/ml.
Enheptin	0·207 ±0·04 ml.	$2{\cdot}70 \times 10^9$/ml.
Enheptin-A	0·242 ±0·05 ml.	$3{\cdot}70 \times 10^9$/ml.

Fig. 1.



Effect of feeding Enheptin and Enheptin-A.

Fig. 2.



Effect of feeding Enheptin and Enheptin-A.

Fertility and Hatchability

In order to ascertain any particular effect on fertility and hatchability, the following mating combinations were used:

Treated ♂ × Normal ♀—2 insem./week. for 4 weeks
Normal ♂ × Treated ♀—2 insem./week for 4 weeks
} Eggs from both matings set simultaneously.

Treated ♂ × Treated ♀—Natural matings for 4 weeks, the males being changed every third day.

The results of these matings are particularly interesting, especially those of the Enheptin-fed group (Table 5). There was a

slight reduction in fertility in the group Treated ♂ × Normal ♀ and the numbers of chicks dying from the 96th hour onwards were considerably greater in this group than in the other combinations. When the same males were mated naturally with treated females such marked differences in embryonic mortality did not occur and this suggested that Enheptin influenced not only sperm density but also sperm viability. In the natural matings it follows that during increased frequency of mating (only 4 to 5 females in lay) there is always a supply of 'fresh' viable sperm in the oviduct. It was apparent that even inseminations undertaken twice weekly with semen from treated males failed to provide sufficient viable sperm to ensure high fertility with subsequent vigorous embryonic development. No detailed observations were made on the effect of Enheptin on embryonic mortality. It appeared, however, from the individual candling at the 96th hour, that the embryos had failed to develop to the same extent as the controls, and when the eggs were candled on the 18th day there was extensive embryonic mortality in this group. On the other hand, Enheptin-A had no adverse effect on fertility, early embryonic development or hatchability.

TABLE 4

EGG PRODUCTION

Groups of 22 pullets fed Enheptin or Enheptin-A from 4 weeks of age

Month	*Controls*	*Enheptin*	*Enheptin-A*
February	158 [12]	38 [4]	173 [14]
March	472 [20]	139 [9]	430 [17]
April	414 [17]	79 [5]	331 [16]
May [20 days]	282 [17]	45 [4]	257 [18]
TOTAL	1326	301	1191

[ ]=No. of pullets in lay.

Histology

Enheptin at 0·05 per cent caused damage to the testes and thus affected normal spermatogenesis. The damage was in the form of patches of atrophy of the seminiferous tubules and fibrosa, some tubules showing few or a complete absence of spermatozoa and spermatids, and regressive changes in spermatocytes, spermatogonia and some Sertoli cells (Fig. 3). In one case the interstitial tissue showed oedema. Less extensive damage of the same type was found in tissue taken from birds fed Enheptin-A, for 10 weeks (Fig. 4). After 26 weeks of continuous feeding with Enheptin-A no testicular lesions were found but after 33 weeks atrophy of single seminiferous tubules was seen (Fig. 6).

TABLE 5

FERTILITY AND HATCHABILITY

	(*a*) *Normal* ♂ × *Treated* ♀			(*b*) *Treated* ♂ × *Normal* ♀			(*c*) *Treated* ♂ × *Treated* ♀		
	Controls	*Enheptin*	*Enheptin-A*	*Controls*	*Enheptin*	*Enheptin-A*	*Controls*	*Enheptin*	*Enheptin-A*
Eggs Set	410	116	355	222	228	292	398	62	337
Infertile	37	11	18	13	30	14	47	4	77
Edg*	32	6	13	14	11	23	11	2	9
Dis†	34	6	23	19	57	35	52	6	20
Errors in candling (i.e.) embryo alive	3	—	5	—	—	—	3	—	1
Chicks hatched	304	93	296	176	130	220	285	50	230
% Fertility	91·0	90·5	94·6	94·1	86·8	95·2	88·2	93·5	77·1
% Edg	7·8	5·1	3·6	6·3	4·8	7·8	2·8	3·2	2·6
% Dis	8·4	5·1	6·6	8·5	25·0	12·0	13·0	9·7	5·9
% Hatch E.S.	74·1	80·0	83·4	79·3	57·0	75·1	71·6	80·6	68·2

(*a*) and (*b*) Inseminated 2 × /wk. with 0·05 ml. undiluted semen.
*Embryonic mortality to 96 hrs.

(*c*) Mated naturally.
†Embryonic mortality 5 days to hatching.

In tissue taken from birds six weeks after the discontinuance of Enheptin and Enheptin-A, patches of atrophy of the seminiferous tubules with a reduction in diameter of the tubules and scanty numbers of spermatozoa were still to be seen. At nine weeks similar lesions together with irregular changes in spermatids were seen only in birds fed Enheptin (Fig. 5). Twelve weeks following discontinuance of Enheptin, atrophied tubules were still seen, but in other tubules scanty numbers of spermatozoa were present.

CONCLUSIONS

Enheptin (2-amino-5-nitrothiazole) and Enheptin-A (2-acetyl-amino-5-nitrothiazole) were fed at preventive levels for periods of 10 and 33 weeks to S.C.W.L. male fowls and for 33 weeks to females. Enheptin was shown to have a marked effect on spermatogenesis and egg production. Although spermatogenesis did not entirely cease, semen was less dense and had a slightly decreased fertilizing ability. There was, however, a marked increase in embryonic mortality unless such males mated frequently to ensure a constant supply of viable sperm in the oviduct. Enheptin-A had no adverse effect on egg production, fertility or embryonic mortality. Semen production and density were slightly reduced owing to patches of atrophy of the seminiferous tubules. Some tubules showed an absence of spermatids as well as decreased numbers of spermatocytes, but these changes were not considered sufficiently great to cause a reduction in fertility and hatchability. Nine weeks after discontinuance of Enheptin-A the testes had returned to normal. Twelve weeks after the discontinuance of Enheptin, the testicles still showed some atrophied seminiferous tubules but in other tubules there were scanty numbers of spermatozoa.

The results of these experiments suggest that Enheptin-A is to be preferred to Enheptin in the treatment of histomoniasis because of the almost complete lack of toxic effects on the reproductive capacity of both sexes.

ACKNOWLEDGMENTS

We should like to express our sincere appreciation to Miss P. Reynolds and Mr. C. Hart for their technical assistance; to Mr. J. Binstead for incubation of eggs and to Mr. W. G. Orbell for taking the photomicrographs. Our thanks are also due to Dr. C. Horton-Smith for his helpful suggestions in preparation of the manuscript and to Mr. S. F. J. Hodgman, M.R.C.V.S. for permission for one of us (G.S.) to work on this project.

The Entramin and Entramin-A were kindly supplied by Messrs. May & Baker, Ltd., Dagenham, Essex.

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Fig. 3.

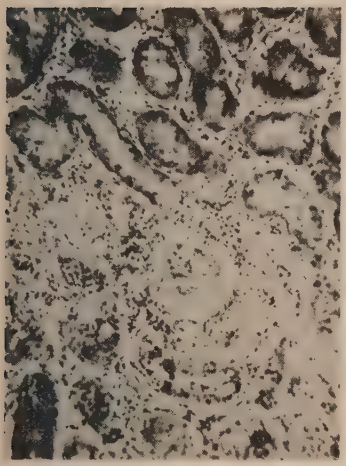

Fig. 4.

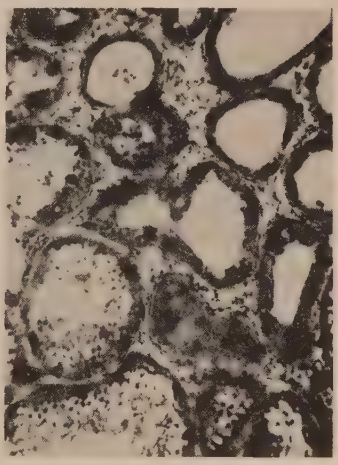

Fig. 5.

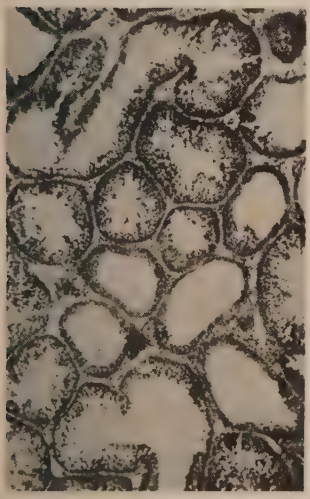

Fig. 6.

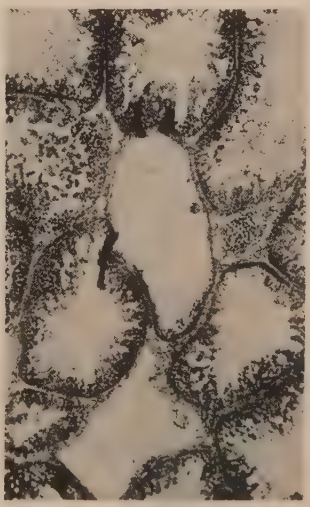

Fig. 3. Testes immediately after feeding Enheptin for 10 weeks. The lower half of the section shows atrophy of the seminiferous tubules and fibrosa. ×200.

Fig. 4. Testes immediately after feeding Enheptin-A for 10 weeks. The tubules show regressive changes: in some only spermatogonia are present, in others spermatogonia and spermatocytes. A normal arrangement of cells though with scanty numbers of spermatozoa is seen in one tubule. ×100.

Fig. 5. Testes 9 weeks after the discontinuance of Enheptin showing some atrophy of tubules. ×100.

Fig. 6. Testes after 33 weeks continuous feeding of Enheptin-A. The tubules show normal numbers and arrangement of cells. Only single tubules are atrophied. ×100.

All sections stained with haematoxylin-eosin.

To face page 194.

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ANAEMIA AND EOSINOPHILIA ASSOCIATED WITH HELMINTHIASIS IN YOUNG HORSES

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INTRODUCTION

The effects of nematode parasites on the blood picture of man and animals have been reviewed by Craig and Faust (1943), but these authors were unable to give any data relating to the horse. The genus *Strongylus,* in which are included the most important parasites concerned in our work, is not mentioned. Von Brand (1952) discussed the mechanism of anaemia produced by infestation with various species of nematodes in man and von Bonsdorff (1956) has reviewed the human anaemia following *Diphyllobothrium latum* infestation. No detailed discussion of anaemia associated with parasitism as it occurs in the horse has been found. Wetzel (1952) mentions an anaemia following experimental infestation with *Strongylus edentatus* in one horse. There has not been any detailed description of the distribution of eosinophil leucocytes in the host tissues in Equidae parasitised by nematodes. Eosinophilia of peripheral blood has long been associated with intestinal parasites in all species including man (Young, 1935; Craig and Faust, 1943). No work upon eosinophil distribution in equine bone marrow has been published.

In the experiment to be described two groups of crossbred ponies were used; one was regularly dosed with anthelmintics and the other was left to acquire naturally a considerable parasitic burden. The parasitised group developed anaemia and a change in the numbers and distribution of eosinophil leucocytes.

METHODS

In August, 1954, ten cross-bred pony foals, aged from two to four months, were selected and divided into two groups. One group of six animals was segregated and placed immediately upon a newly-sown three acre pasture which had not previously been grazed. This group of ponies was maintained as nearly as possible worm-free; the dams of the dosed group had been regularly dosed during the period of lactation to reduce the number of fertile nematode eggs in their faeces. The remaining four animals were placed on a well grown old pasture of four acres, known to be carrying a large number of third stage infective strongyle larvae. No effort was made to control the helmintic burden of these foals or of their dams. Additional feeding of hay in winter was ad lib. in both groups.

The ponies in both groups were weaned at five to six months of age and those in the control group were dosed with anthelmintics according to the schedule given in Table 1. The ponies were bled and faecal samples were taken fortnightly during the thirteen months over which

the observations extended. Each month, from October onwards, bone marrow biopsies were made from the tuber coxae of each of the ten ponies according to the method of Archer (1954 a).

TABLE I

SCHEDULE OF ANTHELMINTIC DOSING OF CONTROL (DOSED) GROUP

Each animal received the following quantities of anthelmintic on the dates shown and in each case administration was by stomach tube.

1954	
September 14th	20 g. Piperazine Adipate
October 25th	25 g. Piperazine Adipate
1955	
January 17th	30 g. Piperazine Adipate
March 22nd	30 g. Phenothiazine
May 11th	50 g. Piperazine Adipate
July 4th	80 g. Piperazine Adipate

Peripheral blood was taken by a needle and paraffined all-glass syringe from the jugular vein and immediately decanted into "Sequestrene" (Archer, 1954 b). Records were made of erythrocyte sedimentation rate (Wintrobe tubes), haematocrit, haemoglobin, red cell count, white cell count, eosinophil count (method of Discombe, 1946) and, from a stained smear, differential white cell count. Bone marrow samples were examined for total nucleated cellularity, eosinophil cellularity and, from stained smears, differential counts were made from which the myeloid-erythroid ratios were calculated.

Faeces samples were examined for the presence of helminth eggs by the Clayton Lane Salt Flotation technique and the Stoll Dilution Count. Identification of eggs of the super-family Strongyloidea was carried out by hatching them and examining the third stage infective larvae.

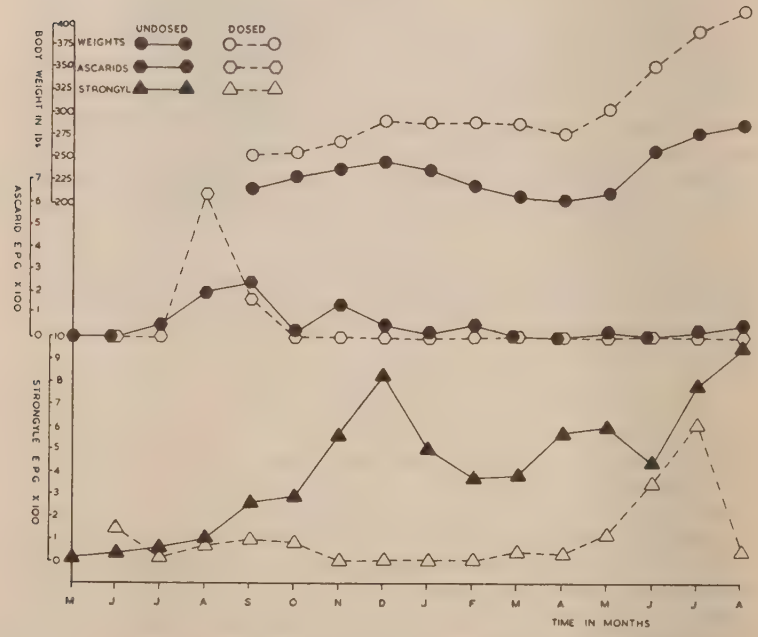
RESULTS

Rate of growth of the parasitised animals as measured by body weight showed a reduction of some 30 per cent as compared with the controls over the period of observation. The curves in Fig. 1 show that the control animals gained weight more rapidly, as compared with the parasitised ones, between April and August 1955. In August, 1955, the two groups exhibited a most marked clinical contrast, the parasitised animals showed staring coats, stunted growth and, indeed, some degree of debility. This clinical difference could be attributed to the degree of parasitism suffered by the undosed group.

The changes in eosinophil content of both bone marrow and peripheral blood are demonstrated graphically (Fig. 2). No effect attributable to ascarids was demonstrable in either group; these helminths could apparently be discounted from an assessment of the haematological response to parasitism in this experiment, where the ascarid burden was relatively light. Strongyle helminths, on the other hand, were associated with a number of changes in both

erythrocytes and leucocytes, especially the eosinophil leucocytes. The general curves of the percentage eosinophil leucocyte content of the bone marrow, the eosinophils of peripheral blood and the number of strongyle eggs in the faeces bore a strong resemblance one to another (compare Figs. 1 and 2). There was no time lag between points of maximal eosinophilia and of faecal strongyle eggs. The anthelmintic treatment given to the control group would so far as is known, have no effect upon migrating larvae; but it should be pointed out that Brown and Chan (1955) found that piperazine hydrochloride administered orally to mice, before and after feeding them infective *Ascaris suum* eggs, did prevent hatching larvae from invading the blood stream and the lungs. The eosinophilia of blood and marrow seen in the undosed group fell considerably when the strongyle egg counts of this group decreased from January onwards.

Fig. 1.

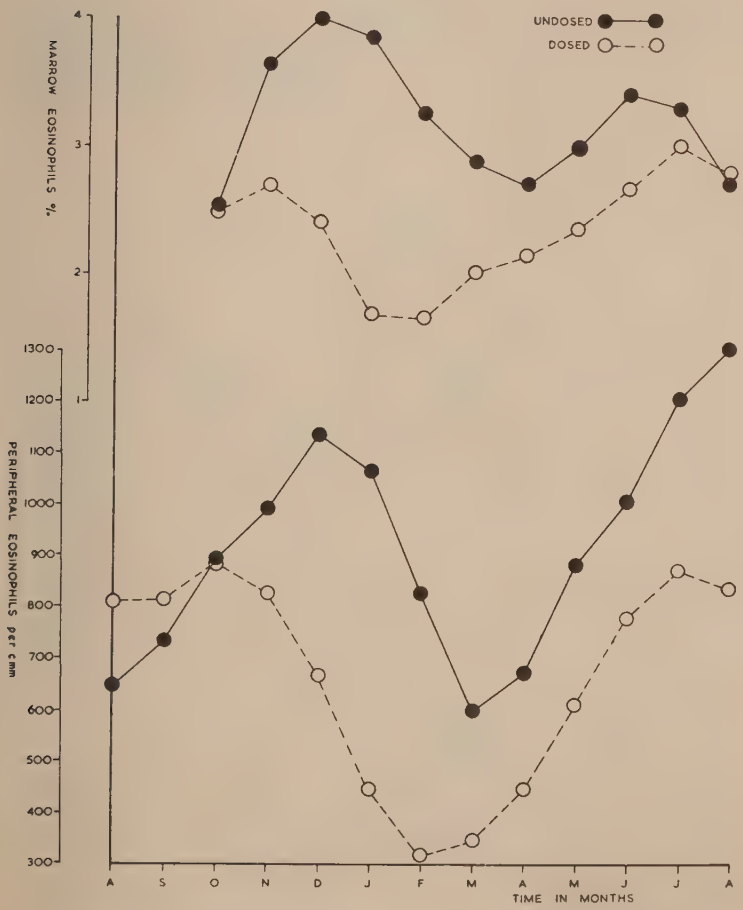

UNDOSED
DOSED
WEIGHTS
ASCARIDS
STRONGYL
BODY WEIGHT IN lbs
ASCARID E P G X 100
STRONGYLE E P G X 100
M J J A S O N D J F M A M J J A
TIME IN MONTHS

Average weight and egg counts of the two groups of ponies during the period of observation.

The average total leucocyte counts for the two groups showed lower figures in the parasitised group than in the control (dosed) group. If these figures were analysed by plotting the numbers of circulating neutrophils and lymphocytes separately (Fig. 3) it could be seen that there was a decrease of some 50 per cent in the

circulating lymphocytes of both groups from August to April whereas there was no great change in the neutrophil count of either group.

Fig. 2.

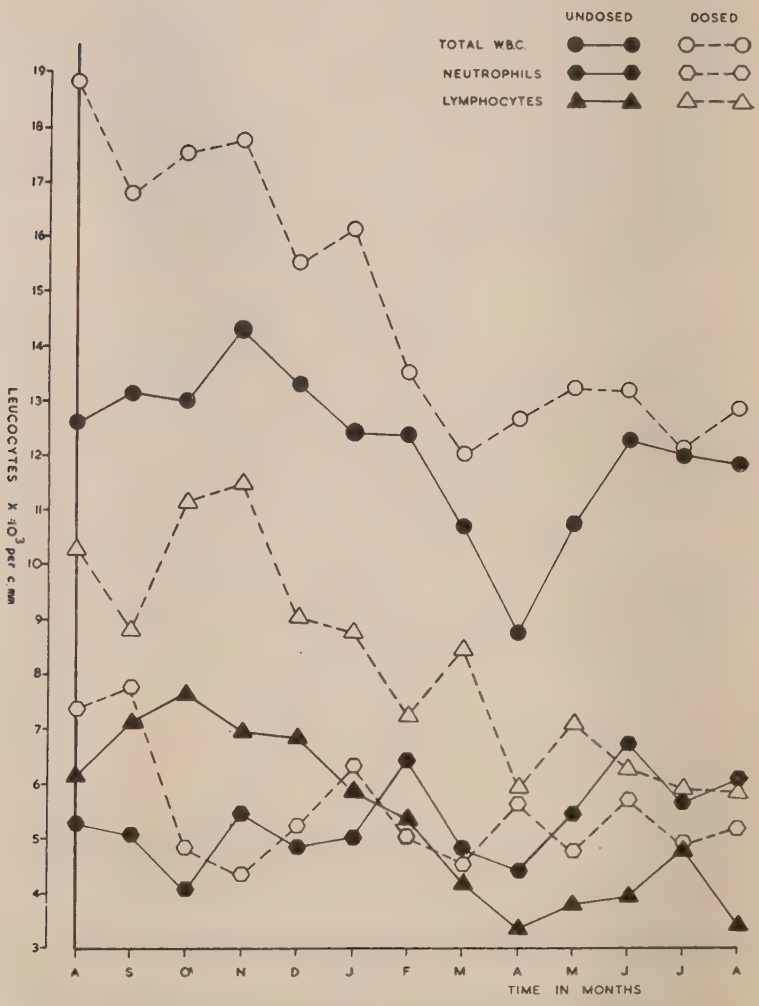


Average percentage eosinophil content of bone marrow, and average circulating (peripheral) eosinophils per cu. mm. during the period of observation.

Anaemia developed in the animals of the parasitised group during the winter and progressed to a maximum during April. The MCHC, MCV and MCH remained practically constant throughout the whole period of observation in both groups. The anaemia, then, was normocytic and normochromic (Fig. 4). There was a striking

recovery in the parasitised group after April which was clearly correlated with the onset and growth of the spring flush of grass in 1955.

Fig. 3.

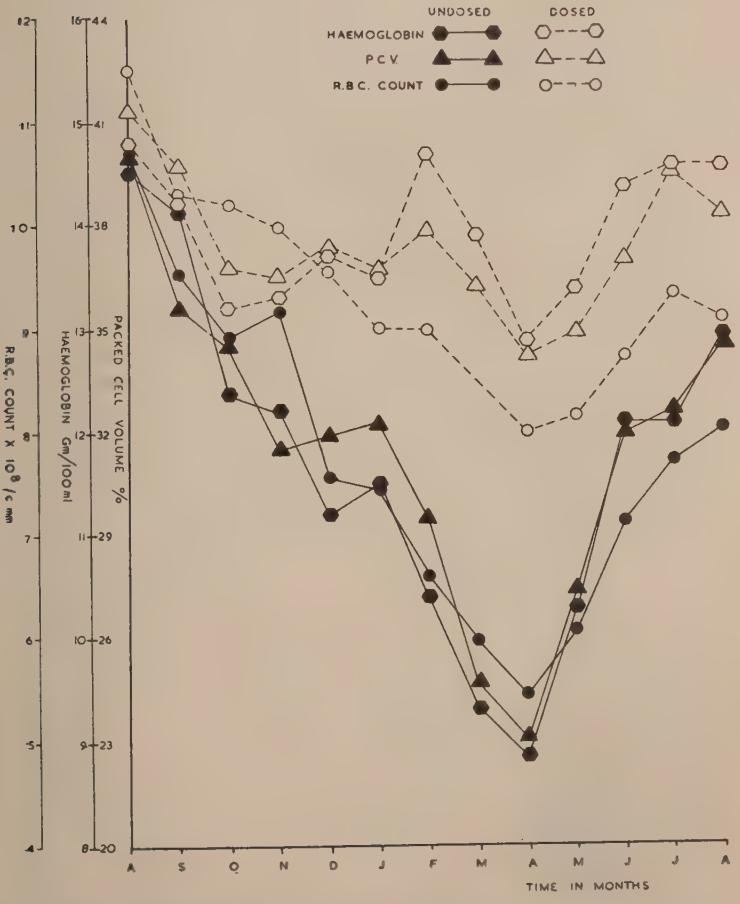


Average total leucocytes, neutrophils and lymphocytes during the period of observation.

The myeloid erythroid (M/E) ratios of the two groups are shown in Fig. 5. The undosed group showed a marked increase in the

M/E ratio, maximal in March and April, when the peripheral erythrocyte count was minimal. The undosed group showed little increase in the ratio and little decrease in red blood cell count (11 down to 8×10^6 per c.mm. as opposed to a reduction from 11 down to $5{\cdot}5 \times 10^6$ per c.mm. in the undosed group). In other words, the bone marrow became more myeloid as the erythrocyte count fell and this was caused by a decrease in erythropoietic elements of the bone marrow, not by an increase in the myeloid.

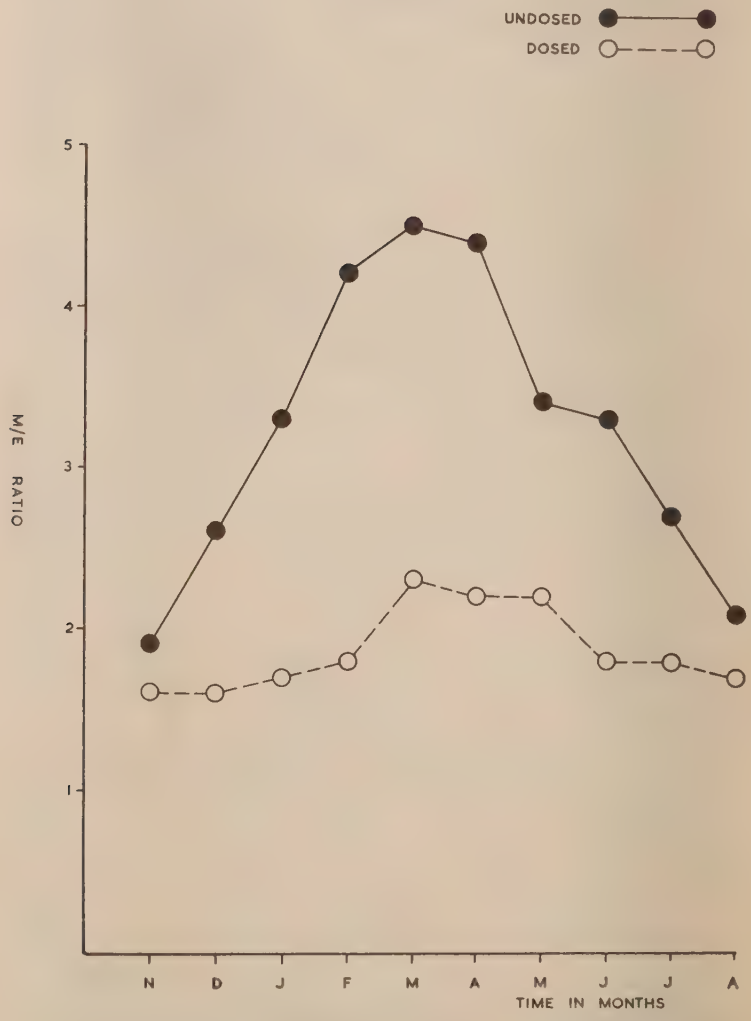
Fig. 4.



Average haemoglobin, P.C.V. and erythrocyte counts during the period of observation.

Horses are probably born free of nematode parasites and acquire infective strongyle larvae and ascarid eggs from their surroundings during the first few days of their lives. These parasites undergo migrations around the body which vary according to the species under consideration. Eventually the larvae return to the gut, reach sexual maturity and commence egg-laying. Although

Fig. 5.



The myeloid/erythroid (M/E) ratios during the period of observation.

nematode eggs are often seen in the faeces of foals during the first four weeks of their lives, they arise from the coprophagic habits of the young animal. The first true signs of the establishment of a nematode population are seen at about four weeks of age when the eggs of *Strongyloides westeri* may be found; at about 12 weeks the eggs of *Parascaris equorum* appear and these are followed at 14 weeks by the appearance of the first strongyle eggs which belong to the genus *Trichonema* (Russell, 1948).

If nematode parasites in horses are responsible for any changes in the blood or bone marrow, such changes would be expected to develop as the parasitic population becomes established in the host. It is known that in adult horses kept at pasture there is a definite seasonal fluctuation in the number of strongyle eggs passed (Poynter, 1954) and it is likely that this fluctuation is related, at least in part, to variations in the numbers of individuals comprising the parasitic population. Russell (1948) studied the development of the helminth population in thoroughbred foals and noted that the number of strongyle eggs gradually increased up to the 56th week of age. In the pony foals used in this study, which were kept at pasture, there was a gradual increase in the average strongyle counts of the four untreated animals which persisted until December 1954, and then fell the following spring. These animals were foaled in May and June and showed a peak in the number of strongyle eggs in their faeces during December, 1954, when 28 weeks old. This is in contrast to Russell's observation and is probably related to the different methods of husbandry, for thoroughbred foals are kept under much less rigorous conditions than the ponies used in this study.

It will be noted from Fig. 1 that the average strongyle egg count of the untreated group fell during the winter months January and February, after which a spring rise occurred similar to that recorded in sheep by Taylor (1935), Morgan and Sloan (1947), Cushnie and White (1948) and Morgan, Parnell and Rayski (1950). In June, 1955, a slight decrease in egg production was noted, but this was followed by a summer rise.

The summer rise started three months after the commencement of the spring rise (Fig. 1). It may be that the spring rise was referable to an increase in the rate of egglaying of existing parasites, as reported in sheep by Spedding and Brown (1956), this leading in turn to an increased infective larval population which gave rise subsequently to the summer rise following upon an increase in actual worm burden. The gap of three calendar months would then be a measure of the time taken for the infective larvae of the genus *Trichonema* formed during the spring rise to infect their hosts, undergo development, and contribute as adults to the summer rise. It should be mentioned that all the ponies, apart from one in the dosed group, were infected with *Strongyloides westeri* for the early part of their lives. There is no recognised treatment for this parasite and indeed it is usually non-pathogenic. Since both groups were

similarly infested with this worm it is not possible to consider it in relation to the changes in the blood pictures of the two groups.

In general, the control group of animals maintained relatively free from helminth parasites by the use of a new pasture, together with regular anthelmintic treatment, showed little change during the year of observation in M/E ratio, erythrocyte numbers or in helminth eggs in faeces. The counts of eosinophil leucocytes in the dosed group of animals were consistently lower than those seen in the undosed group, except in August and September, 1954, when helminth burdens were not fully established. The undosed group showed a marked normocytic, normochromic anaemia, together with erythroid hypoplasia of the bone marrow, which reached a maximum during April 1955. The increase of eosinophil leucocytes, as also the anaemia, ran closely parallel to the helminth burden.

Parasitological Findings at Post-mortem Examination

At the termination of the experimental period two animals from the undosed group and one from the group kept under conditions designed so as to reduce parasitic burdens to a minimum were slaughtered. This provided accurate information about the helminth populations and allowed an assessment to be made of the number and species of worms which provoked the effects reported in the earlier part of this paper. The results showing the number and species of parasites (Table 2), indicates that the animals from the

TABLE 2

PARASITIC BURDENS OF THREE EXPERIMENTAL PONIES

Location	*Parasite*	*U.1.*	*U.2.*	*D.1.*
Stomach ..	*Gastrophilus* spp.	61	0	105
Small Intestine	*Anoplocephala perfoliata*	0	0	19
Caecum ..	*Trichonema* spp.	1	13	0
	Strongylus edentatus	1	8	0
	Strongylus vulgaris	64	124	1
	Triodontophorus spp.	0	27	0
	Oxyuris equi	1	0	0
	Anoplocephala perfoliata	0	0	92
Colon ..	*Trichonema* spp.	725	1331	80
	Strongylus edentatus	91	140	10
	Strongylus vulgaris	57	16	1
	Triodontophorus spp.	127	36	0
	Poteriostomum spp.	408	287	2
	Gyalocephalus capitatus	0	8	1
	Oesophagodontus robustus	0	4	0
	Oxyuris equi	103	162	0
Rectum ..	*Trichonema* spp.	3	0	0
	Strongylus edentatus	1	0	0
	Poteriostomum spp.	1	0	0
	Oxyuris equi	5	2	0

undosed group possessed a helminth burden differing greatly in both species composition and quantity from that in the dosed animals. Animals U.1. and U.2. were from the undosed group whilst D.1. was from the dosed group. Although ascarid eggs were present in the faeces of some animals of the undosed group until the end of the observations (Fig. 1), they were not seen in animals U.1. and U.2. and it is, therefore, not surprising that no ascarids were recovered at the post-mortem examinations of these animals. Histological examination of the bowel wall revealed an extensive eosinophilia which was not limited to those areas where parasites were found.

DISCUSSION

The anaemia produced in cross-bred ponies by nematodiasis was normocytic and normochromic, but was none the less well-marked. The bone marrow exhibited erythroid hypoplasia, but differential counts showed no shift to right or left of the erythroid elements. The hypoplasia appeared to affect erythroid cells in all stages of development, but the myeloid elements were not involved. There was no evidence of macrocytosis of the bone marrow, nor has any evidence yet been found that macrocytic erythropoiesis can take place in the horse. In fact, apart from the erythroid hypoplasia, no positive abnormality could be detected in the cytological picture of bone marrow from the parasitised animals.

The depression of bone marrow activity which occurred in the parasitised ponies might be due to the absorption of vitamin B_{12} by the parasites in the lumen of the bowel, as has been recorded in chickens (Sadun, Totter and Keith, 1949). On the assumption that the intake of vitamin B_{12} was marginal or limited in the experimental ponies, a deficiency might arise in the presence of many helminth parasites. Alternatively, it might be that the parasites elaborate some toxic substance which exerts a direct depressing action upon the erythroid elements of the bone marrow. There was, however, a stimulating effect upon eosinophil granulopoiesis. Clinically, vitamin B_{12} frequently has a most marked tonic effect when given empirically and it is quite possible that this effect may be produced by the reduction of the helminth-induced deficiency of the vitamin. Grunsell (1955), in a report on anaemia of sheep associated with parasitism, demonstrated a reduction in bone marrow erythroid tissue and a resulting reduction in the circulating numbers of erythrocytes. His findings, therefore, are substantially the same as ours in the horse.

The presence of helminths in the bowel led to an increase in the number of eosinophil leucocytes within the wall of the gut, as shown at post mortem examination. There was, in general, eosinophilia of the blood and of the marrow at corresponding times and to equivalent extents, but the eosinophilia in the peripheral blood varied greatly in different individuals. An increase in the

percentage number of eosinophils of bone marrow indicates an increase in demand for these cells, but does not automatically produce peripheral eosinophilia. Heavy eosinophil infiltration of the bowel wall in parasitised animals is the rule, but is not limited to those areas where helminths can be identified. The cells removed from circulating blood by this means may well be reflected by an increase in bone marrow eosinophils, the peripheral blood being merely a means of conveyance of these cells from bone marrow to tissues. There is evidence that the duration of life of eosinophil granulocytes in peripheral blood is only about four hours (Dustin and de Harven, 1954; Archer, 1956 b) and there is also evidence that peripheral eosinopenia can be induced within a few hours by shock (Selye, 1949) or by certain histamine-like agents (Åkerblom and Sjöberg, 1938; Archer, 1956b). Examination of a single sample of peripheral blood showing eosinophilia would be quite inconclusive as evidence for parasitism, although it would be suggestive. But a single bone marrow biopsy would be very highly suggestive of parasitism if the eosinophil percentage exceeded about 2·5 and so long as no other reason for eosinophilia of the marrow was suspected. We know of no naturally occurring condition which produces a profound eosinophilia of the bone marrow of the horse.

The correlation of eosinophilia with egg-laying of adult helminths as measured by faecal contents is marked. During October/December, 1954, egg production in the undosed group reached a peak which is reflected by the peripheral eosinophils of the same group. Both curves follow the same general form throughout the remainder of the period of observation. The number of peripheral eosinophils in the undosed group reached a maximum in August 1955 at the same time as the strongyle egg count for this group also reached its maximum. Tissue migration of larvae did not produce any degree of eosinophilia comparable to that apparently produced by stimulation of the bowel wall by adults in the lumen of the alimentary canal. It is, however, quite possible that the eosinophilia of the bowel wall is not a direct consequence of the presence of adult helminths. Indeed, during March and April (1955) and in September and October (1954) both marrow and blood eosinophil levels were particularly low.

No peripheral eosinophilia has been found at any time which is comparable to that seen in man after, for example, infestation with the giant intestinal fluke, where peripheral blood counts as high as 10,000 eosinophils per c.mm. have been reported (Young, 1935).

CONCLUSIONS

Cross-bred ponies infested with various species of strongyle helminths developed a normocytic, normochromic anaemia with erythroid hypoplasia of the bone marrow.

The ponies also showed an eosinophilia of the peripheral blood

and bone marrow and there was evidence that this eosinophilia was related to the population of adult strongyle helminths in the lumen of the bowel.

A temporary and light infestation with *Parascaris equorum* produced no demonstrable effect upon the peripheral blood or bone marrow.

It is suggested that the peripheral erythrocytopenia and erythroid hypoplasia of bone marrow may be associated with a derangement of absorption from the bowel directly referable to the parasitic burden.

ACKNOWLEDGMENTS

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SEROLOGICAL INVESTIGATION OF MYXOMA AND FIBROMA VIRUSES

I. Complement-fixation Test

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INTRODUCTION

Van Rooyen and Rhodes (1938) were able to demonstrate complement-fixing antigen in the deposit of a suspension of myxoma-infected rabbit testicles after centrifugation. Hyde (1939) demonstrated the antigen in infected oedematous skin. Fenner and Woodroofe (1953) detected the antigen in myxoma-infected rabbits from the time of inoculation until 9 days later at the site of inoculation, in the secondary lesions, in the regional lymph nodes and in the blood serum.

In the early stage of our investigation, the infected chorio-allantoic membrane of chick embryos was used as the source of stock myxoma-virus antigen for diagnostic purposes, following the technique first described by Lush (1939) and modified by Fenner and Woodroofe (1953, 1954). In September 1954, a comparative study was made of the egg antigen and of material prepared directly from infected rabbits to determine whether the latter could be used as stock antigen for testing routine serum samples. The technique and methods were those described by Mansi (1955).

MATERIAL AND METHODS

Myxoma-virus antigen. Different tissues were collected from infected, recovered, immune and normal rabbits. Rabbits were infected by intradermal or subcutaneous inoculation of myxoma virus. A fully virulent strain ("Cornwall") and an attenuated strain Mx/E/155 (Hudson and Mansi, 1955) were used. A 10 per cent suspension was prepared by grinding the tissues in a Ten Broeck grinder, using normal saline solution as diluent.

Myxoma-egg-virus antigen (C.A.M.) was prepared from infected chorio-allantoic membranes harvested three days after inoculation and diluted 1/8 in normal saline.

Fibroma-virus antigen was made in a similar way to that described for the myxoma rabbit tissue antigen. The rabbits were inoculated with the Boerlage strain of Shope's fibroma virus.

Myxoma-immune rabbit sera were collected from myxoma-recovered rabbits, usually 1 to 2 months after infection.

Fibroma-immune rabbit sera were taken from fibroma-vaccinated rabbits, 1 to 2 months after inoculation.

Normal (negative) controls. The spleen or testicles of normal rabbits were treated in the same way as the tissues of infected rabbits to furnish negative controls. Sera collected from normal, susceptible rabbits, were used as negative serum controls.

Inactivation of rabbit sera. Sera were diluted 1:4 in normal saline and then heated at 64°C. for 20 minutes.

Complement. Freeze-dried complement was used throughout. Sera of suspected immune rabbits were first tested by the complement-dilution method. Strongly positive sera were then titrated using a single dose of complement.

Freeze-dried complement, sera and antigens provided suitable materials to carry out routine diagnostic tests and reduced considerably the amount of work involved.

RESULTS

Comparison between Egg (C.A.M.) and Infected Rabbit Antigens

It was found (Table 1) that C.F. antigen could be prepared easily from infected rabbit's tissues. Such antigen was of a higher titre than that prepared from chick embryos. A titre of 1/638 to 1/9163 was obtained without difficulty, depending on the tissue collected, while the highest recorded titre prepared from the C.A.M. was 1/128. It was also found that the amount of C.F. antigen prepared from the oedematous lesions of one infected rabbit was equal to that obtained from 16 dozen infected embryos. The virus grew only on the C.A.M. and no traces of antigen were found elsewhere in the different tissues of the chick embryo. The myxoma virus antigen was demonstrable in different internal organs of the rabbit as well as in the oedematous lesions, while Shope's fibroma virus antigen was found only in the local lesions at the sites of inoculation.

TABLE 1

COMPARISON OF EGG AND RABBIT ANTIGENS

Source of antigen	*Tissue examined*	*Complement-fixing antigen titre*	
		Average	*Highest*
Chick embryo materials collected 3 days after inoculation	Chorio-allantoic membrance	40*	128
	Other tissues	0	0
Rabbit tissues collected from 17 typical cases 9-16 days after inoculation	Site of inoculation	9,163	25,000
	Eyelid	7,347	32,000
	Spleen	8,307	32,000
	Anal ring	4,057	10,240
	Liver	5,527	40,960
	Lung	2,480	10,240
	Kidney	897	2,560
	Lymph node	638	5,120

*In this and the following tables the figures are reciprocals of the dilutions.

Titration of Myxoma and Fibroma Serum in the presence of Different Concentrations of the Homologous Antigens

It was shown that serum collected from a fibroma-vaccinated rabbit reacted in a higher dilution with fibroma antigen while serum

collected from a myxoma-immune rabbit reacted in a higher dilution with myxoma antigen (Table 2). The titre of antibodies obtained in each was relatively higher when strong dilutions of antigens were used.

TABLE 2

TITRATION OF FIBROMA AND MYXOMA ANTISERA

Virus antigen	*Antigen dilution*	*Titre of antibodies in*	
		fibroma serum	*myxoma serum*
Fibroma	1/100	128	8
	200	64	0
	400	64	0
	800	0	0
Myxoma	1/100	8	2056
	200	8	1028
	400	4	512
	800	4	256
	1600	0	64
	3200	0	16

The Effect of Heat on the Antigen

Tissues were collected from a fibroma and from a myxoma-infected rabbit respectively, ground and antigens prepared. The results of testing such suspensions when fresh, after exposure to heating at 64°C. and after boiling are shown in Table 3.

Taking into consideration that the titre of myxoma antigen was initially much higher than fibroma antigen, the effect of heating is interesting. The reduction of titre was very marked with the former when a sudden drop from 20,480 to 2,560 was recorded. Exposure to further heating and boiling, however, appeared to reduce the antigenic property only gradually until after 120 minutes no further C.F. antigen was detectable. Fibroma antigen was totally destroyed after boiling for 10 minutes.

The normal rabbit antigen in this experiment acted as a control.

Distribution of Myxoma C.F. Antigen in Infected, Recovered and Immune Rabbits

The antigen content varied from tissue to tissue, from one rabbit to another and at different periods of infection. The results (Table 4) indicate this variation. In animals which died of the typical infection, a high concentration of soluble antigen was distributed throughout the body from the 9th to 16th day after inoculation. From the 17th to 20th day the titre declined in the spleen, lymph node, kidney, liver and lung, while it increased in the site of inoculation, the eyelid and the anal ring. In 14 rabbits which recovered from a mild nodular attack and were killed 21 to 37 days after inoculation, the soluble antigen had disappeared from the lung,

liver and lymph node. The titre declined considerably in other tissues especially in the spleen, kidney and anal ring; it was still high in the nodule at the site of inoculation and the eyelid. Four rabbits killed 38 to 94 days after inoculation when they had almost completely recovered, showed no soluble antigen in their tissue. One rabbit was killed at 74 days after an unusually chronic attack; no soluble antigen was present in the lymph node, the kidney, or the lung, but there were low titres in the spleen (1/160), in the liver (1/640) and in eyelid and anal ring (1/1280). Fibroma-vaccinated rabbits which resisted a myxoma challenge and were killed 21 days or more after challenge showed no C.F. myxoma antigen in their tissues.

TABLE 3

THE EFFECT OF HEAT ON THE VIRUS ANTIGEN

Virus antigen	*Treatment*	*Myxoma serum 50*	*Fibroma serum 50*	*Normal serum 50*
Myxoma ..	Unheated	20,480	10	0
	64°C 10 minutes	2,560	0	0
	64°C 30 „	640	0	0
	64°C 60 „	640	0	0
	64°C 120 „	320	0	0
	Boiled 10 „	160	0	0
	Boiled 30 „	160	0	0
	Boiled 60 „	80	0	0
	Boiled 120 „	0	0	0
Fibroma ..	Unheated	160	320	0
	64°C 10 minutes	40	160	0
	64°C 30 „	20	40	0
	64°C 60 „	0	40	0
	64°C 120 „	0	40	0
	Boiled 10 „	0	0	0
	Boiled 30 „	0	0	0
	Boiled 60 „	0	0	0
	Boiled 120 „	0	0	0
Normal rabbit tissue	Unheated	0	0	0
	64°C 10 minutes	0	0	0
	Boiled 10 „	0	0	0

There was a close correlation between the antigenic contents of the local lesions, eyelids and spleen in 88 experimentally infected rabbits both at the height of infection and at death. In only one of a group of 15 rabbits which were killed between 21 and 30 days after inoculation was there a considerable titre of C.F. antigen in the spleen, while in 7 cases it was still demonstrable in the eyelid and local nodules.

The brains of 21 rabbits, 19 of which were fatal cases in which an average titre of 1/8307 C.F. antigen was present in the spleen and 2 were recovered rabbits with little residual titre (40 to 160), were tested for the presence of C.F. antigen. Two of the 19 brains had

TABLE 4

DISTRIBUTION OF MYXOMA ANTIGEN

Clinical condition	*Days after inoculation*	*Number of rabbits examined*	*C.F. anti-body titre (average)*	*Distribution of C.F. antigen (average)*							
				Site of inoculation	*eyelid*	*lung*	*liver*	*kidney*	*lymph node*	*anal ring*	*spleen*
Early infection (acute) ..	6-7	5	0	3093	920	1053	1226	156	613	933	3256
Died of typical infection	9-16	17	0	9163	7347	2480	5527	897	638	4057	8307
Died of chronic infection	17-20	4	0	16000	17706	200	2100	140	70	7600	5600
Killed when recovering from atypical infection	21-37	14	1216	7591	903	00	00	16	00	118	13
	38-94	4	416	640	00	00	00	00	00	00	00
	74	1	1024	1280	1280	00	640	00	00	1280	160
Healthy (immune) ..	21-70	10	474	00	00	00	00	00	00	00	00

very low titres of 10 and 80 respectively. The two brains collected from recovered rabbits contained none.

Distribution of C.F. Antigen after Intracerebral Inoculation of Neuromyxoma

The results of these experiments showed that the myxoma C.F. antigen was not detectable in the body of infected rabbits before the 96th hour after intracerebral inoculation (Table 5). It was also observed that the antigen titres in the different tissues were not as high as in the tissues of rabbits infected with virulent myxoma virus (Table 4). There was a close correlation between the mildness of the attack and the rate of recovery, and the decline in the titre of myxoma C.F. antigen. It was only possible to demonstrate the presence of the antigen in the blood at 144 hours. No rabbit killed during the first ten days after inoculation showed C.F. antibodies in its serum.

Complement-fixing Antibodies

Among 981 rabbit sera examined, 677 were collected from wild and 304 from laboratory rabbits. In the first group 28 samples were collected from rabbits showing typical symptoms and none of these showed any C.F. antibodies; whereas 26 of 28 samples from rabbits showing evidence of an atypical infection, some of which had almost recovered, showed antibodies. The remaining 621 samples were from clinically healthy wild rabbits: 165 contained C.F. antibodies and 456 were negative. Of the 304 laboratory rabbits, 55 samples were collected from animals showing typical myxomatosis after the intradermal inoculation of the "Cornwall" strain of myxoma virus and from which they eventually died in from 9 to 15 days. None of these animals showed C.F. antibodies. Only 4 out of 13 rabbits which died between 16 to 25 days after inoculation showed C.F. antibodies.

Of 24 rabbits inoculated intracerebrally with neuromyxoma virus, 10 were killed 1 to 10 days later and none showed C.F. antibodies. The remaining 14 recovered and developed C.F. antibodies. Of 68 rabbits which recovered after intradermal inoculation of the Nottingham strains (Mx/E/155 and 201) only one failed to show C.F. antibodies in blood samples collected 21 to 70 days after inoculation.

Among 144 rabbits vaccinated with fibroma virus and found to be resistant on challenge with myxoma virus (Cornwall strain), 141 showed C.F. antibodies. C.F. antibodies were usually demonstrable from the 11th day after inoculation. In a few cases, however, they were only demonstrable after 5 to 10 weeks. The titre usually remained high for the first six months, but in a few instances it had fallen by the 3rd or 4th month, while 60 to 72 weeks after inoculation only 7 out of 17 animals were still showing antibodies.

When antibodies were expected to be of a low titre, serum was diluted 1/4 and tested against varying dilutions of complement.

TABLE 5

DISTRIBUTION OF ANTIGEN AFTER NEUROMYXOMA INOCULATION

Hours after intracerebral inoculation	*Symptoms*		*Distribution of c.f. antigen*									*c.f. antibody*
	early	*general*	*brain*	*spleen*	*liver*	*lung*	*kidney*	*lymph node*	*eyelid*	*anal gland*	*blood*	
24	—	—	0	0	0	0	0	0	0	0	0	0
48	—	—	0	0	0	0	0	0	0	0	0	0
72	—	—	0	0	0	0	0	0	0	0	0	0
96	—	—	0	0	0	0	0	0	0	0	0	0
144	+	—	10	40	40	10	5	0	320	—	5	0
168	+	±	5	10	0	0	0	0	160	0	0	0
168	+	Died	40	80	80	40	10	0	320	0	0	0
192	+	±	20	80	320	20	20	0	640	320	0	0
216	+	±	5	10	0	5	20	0	80	20	0	0
240	+	improving	5	0	0	0	10	0	5	0	0	0

DISCUSSION

It was found that myxoma virus antigen prepared from the tissues of infected rabbits was of higher titre than that of the infected chorio-allantoic membranes of chick embryos. Antigens prepared by disintegration, freezing and thawing were devoid of anti-complementary properties.

The use of antigens prepared directly from animal tissues for the complement-fixation test saved time and materials when compared with antigens prepared from chick embryos.

The study of the distribution of the myxoma antigen indicated that from the 7th day after inoculation it was demonstrable in almost every internal organ. Compared with other organs, the brain was a poor source of antigen, even when neuromyxoma virus was inoculated intracerebrally. Antigen persisted in the internal organs until the animal died of the infection or recovered. While in some other virus infections e.g. canine distemper and canine hepatitis viruses, the appearance of C.F. antibodies follows the disappearance of the antigen in the tissue of the infected animal, in the case of myxomatosis it was sometimes possible to demonstrate both antibody and antigen at the same time.

The presence of antibodies to myxoma virus in a rabbit showing lesions would suggest that the animal was recovering. If the lesions consisted of dry scabs, recovery would be almost complete. In many rabbits which showed no clinical evidence of ill health the presence of myxoma antibodies left no doubt that they were immune to the disease. Such antibodies persisted in the circulation for a period of time which varied considerably in different individuals.

Although van Rooyen and Rhodes (1938) found no serological inter-relationship between the two viruses, it appears that there is a low degree of cross-reaction between antigen prepared from fibroma and myxoma rabbit tissues. This confirms the finding of Fenner and Woodroofe (1954) who used myxoma antigen prepared from infected C.A.M. membrane and fibroma antigens prepared from rabbit tissues.

CONCLUSIONS

Complement-fixing antigen of myxoma virus was prepared directly from infected rabbits for diagnostic purposes. Such antigen was of a considerably higher concentration and much cheaper to produce than that prepared from chorio-allantoic membranes of infected chick embryos.

A study of the distribution of the antigen in different tissues of dead, recovered and immune rabbits was made.

A comparative study of myxoma and fibroma virus antigens was carried out.

ACKNOWLEDGMENTS

I wish to thank Dr. Stableforth for his encouragement and for permission to publish this work, Mr. J. R. Hudson for his advice and interest, and Mr. A. A. King for technical assistance throughout this work.

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CONTAGIOUS PUSTULAR DERMATITIS

II. Pathological Histology

By

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INTRODUCTION

Previous work on the histology of skin changes in contagious pustular dermatitis (C.P.D.) has been confined to the study of gross changes and to the finding of a sequence similar to that occurring in vaccinia. In the present investigation, these changes were re-studied with particular reference to degenerative changes in epidermal cells and the formation of inclusions. These cells were found to react characteristically by undergoing reticular degeneration (Unna, 1894) with the formation of cytoplasmic inclusions. Contrary to previous reports in the literature, no 'balloon' degeneration of the epidermal cells was seen in C.P.D.

MATERIAL AND METHODS

Glover (1928) described the histology of C.P.D. in lambs inoculated with the U.F. strain which was also used in the present studies. In order to obtain discrete lesions, lambs were lightly scarified on the inside of thighs and on the bare area in front of the inguinal region with a sharp needle and inoculated with a dilute suspension of elementary bodies. Pieces of the inoculated skin were excised under local anaesthesia at frequent intervals and fixed after stretching on stiff paper. The fixative used was Heidenhain's 'Susa' mixture but occasionally formol-calcium (Baker, 1945) or five per cent formol-saline were employed. Sections were stained with Delafield's haematoxylin-eosin and occasionally with Heidenhain's iron-alum haematoxylin, Mann's methyl-blue eosin, Giemsa, Weigert's fibrin stain and for Feulgen reaction.

RESULTS

Histology of the Normal Sheep Skin

While the general features of the histology of sheep skin have been described in the relevant textbooks (Trautmann and Fiebiger, 1952; Hieronymi, 1924) no detailed information on keratinization was found in the available literature. This information was considered to be important as the normal degenerative changes occurring in the process of keratinization have not infrequently been confused with those due to disease; moreover, a study of the epidermal cells in the particular area of skin and under conditions of fixation, etc. employed was necessary for comparison.

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The epidermis on the inside of the thigh of sheep was found to be thin and to consist of three layers, which from the deepest outward were: (1) the prickle-cell layer of Malpighi (stratum germinativum or spinosum); (2) the granular layer (stratum granulosum) which was not continuous and was represented by a few cells containing granules; and (3) the horny layer (stratum corneum). The clear layer (stratum lucidum) was not obviously distinguishable.

The Malpighian layer, which is of principal interest in the pathology of C.P.D., consisted of three or four layers of cells. The basal layer comprised cylindrical cells with oval nuclei, the long axis of the cell as well as the nucleus being at right angles to the skin surface (Fig. 1). The cytoplasm contained a large number of tonofibrils going in various directions and enclosing granular matter in the meshes. Some of these granules may have been the fibrils in cross section. Vacuoles were not observed in the cytoplasm and fibrils passed out of the cell membrane on all sides, except the basal surface next to the corium, to form intercellular bridges or 'prickles'. The nucleus was oval and contained a uniformly distributed chromatin network and granules some of which lay next to the nuclear membrane. Each nucleus had one (rarely double) nucleolus situated in a variable position and staining partly with basic and partly with acid dyes. Mitotic figures were only rarely seen in the nuclei of cells in the unaffected skin.

Towards the middle of the Malpighian rete, the cells tended to be less cylindrical and more rectangular in outline. The cytoplasm was similar to that in the basal layer and the intercellular fibrils (prickles) were still clearly visible. The nucleus tended to be globular and the chromatin granules had moved towards the periphery (Fig. 2).

In more superficial parts of the Malpighian rete the cells became very much compressed distally and the nucleus again became ovoid, this time, with the long axis parallel to the skin surface. The cytoplasm was crowded with fibrils running parallel to the surface. The nuclear membrane was shrivelled and occasionally fragmented. The chromatin granules had all migrated to it, and here and there, a nucleolus could be seen in the process of extrusion. The nucleolus had become more acidophilic but not very much enlarged. The intercellular bridges were no longer discernible. Between these superficial rete cells and the horny layer there were a few layers of fibrils or collapsed denucleated cells in which acidophilic granules could occasionally be seen (stratum granulosum) and next to this was the horny layer consisting of acidophilic keratin fibrils.

Other parts of the skin and its glands and appendages are not described here as they do not play an important part in the cellular response to C.P.D.

Histology of the Skin Response of Sheep to C.P.D.

The sequence of changes could not be divided into clear-cut papular, vesicular and pustular stages, as at a certain period all could be seen in a single eruption, the centre being at a more advanced stage than the periphery.

Sections of the skin excised 24 hours after inoculation showed intercellular oedema evidenced by widening of the lymph channels and slight infiltration of the corium with mononuclear cells. It was

not certain, however, whether these changes were due to the virus or to the trauma of inoculation by scarification. The first well-marked changes were seen after 48 hours, even though no naked-eye changes were discernible at this stage. In places, cells of the granular layer and some superficial ones of the Malpighian layer became swollen. The cytoplasm contained vacuoles and there was shrinkage of the nucleus, which may have been partly due to normal keratinisation. There was no definite increase in the number of layers of cells at this stage, but the epidermis appeared to be thickened as a result of swelling of cells which are normally flattened. The corium showed infiltration with mononuclear cells, congestion of blood vessels and some proliferation of connective tissue.

On the third day, when the first naked-eye symptoms appeared, the cellular response was well marked. The Malpighian rete had proliferated to three or four times its normal thickness and the vacuolation and swelling of cells had proceeded to a deeper level. The cells of the basal layer were enlarged and mitotic figures could be seen in their nuclei. At this stage, cytoplasmic inclusions appeared in some of the cells. The corium was more heavily infiltrated with inflammatory cells in which polymorphonuclears now predominated and the whole lesion tended to spread outwards (Fig. 3).

On the fourth day, the superficial cells in the centre of the lesions were necrosed and there was infiltration with polymorphonuclears and lymphocytes, thus showing the first signs of suppuration. The outer parts of the lesion showed various stages of the earlier changes described above (Fig. 4).

From the fifth to the eighth day the necrosis and suppuration spread outward to the vacuolated cells of the epidermis and the lesion became localised. The superficial necrotic mass mixed with fibrin, cell debris and infiltrating cells and bacteria became demarcated and formed a scab. Healing took place beneath this scab by regeneration of the epithelium in about three weeks when the crusts fell off.

Cellular response in the epidermis. The vacuolation of epidermal cells and appearance of cytoplasmic inclusions in them has been referred to above. These changes occur in the course of degeneration of epidermal cells, the full sequence of which is described below. All these changes could be seen in a 4 or 5-day lesion (Fig. 5).

The first change in the basal cells of the Malpighian layer was an intercellular oedema evidenced by widening of the intercellular lymph channels followed by a marked enlargement, usually up to 5 times the normal size of the cell. The increase in size was proportionately more in the cytoplasm than in the nucleus. The tonofibrils were separated by empty spaces, most probably occupied in life by oedematous fluid, particularly around the nucleus. There was some granular material interspersed among the tonofibrils. The intercellular channels were obliterated and no longer visible. The nucleus

was enlarged, usually not to more than double its normal size. The nucleolus was distinctly enlarged and often surrounded by a halo of empty space, possibly occupied by fluid. The chromatinic granules tended to migrate towards the nuclear membrane, a change occurring much later in normal keratinization. The nucleolus also tended to be more acidophilic and with its increased size and the surrounding halo resembled the intranuclear 'inclusions' reported in varicella (Tyzzer, 1905–6; Rivers, 1926).

In the next few layers, towards the surface, the foregoing change progressed by further recession of tonofibrils leaving a circumscribed clear space around the nucleus. This space sometimes contained some granular matter and enlarged until all the fibrils were pushed to the periphery, thus forming a compact fibroid layer inside the cell membrane. In the cavity so formed, the granular material increased, and in many cells appeared to come together to form a compact body, 4 to 8 μ in diameter and roughly globular, sub-globular, or irregular in shape. This answered the description of the cytoplasmic inclusion given by Saliyi (1939). With haematoxylin and eosin, these bodies stained greyish red and gave a faintly positive Feulgen reaction. In some cells these inclusions appeared to be suspended in a fibrillar stroma. Saliyi (1939) saw these inclusions on the third to fifth days and rarely on the sixth day. In the present investigation, they were found to be abundant on these days but rare ones could be detected up to the eighth day, i.e., as long as the reticular degeneration lasted. The nucleus in these layers shrank and collapsed to discharge the intact or broken nucleolus, which was often seen lying in the cell cavity beside the inclusion body. Groups of cells showing the foregoing changes appeared like a network under the low power of the microscope. Nearer the surface of the epidermis, the cells became compressed distally and the inclusions disintegrated to form a granular mass. The degenerating cells did not become detached from each other and the intercellular septa formed by the cell membrane reinforced with tonofibrils did not break up to form large vesicular cavities as in vaccinia and variola (Benda, 1927; Hieronymi, 1927).

It will be seen that the degenerative change described above corresponded to the reticular degeneration described by Unna (1894) and not to 'balloon' degeneration in which the affected cell does not increase in size, becomes rounded and detached and the nucleus does not collapse, the cell as a whole undergoing colliquation.

DISCUSSION

The characteristic response of sheep skin to C.P.D. virus consists of a proliferation and reticular degeneration of epidermal cells leading to necrosis accompanied by the infiltration of the corium with polymorphonuclear and mononuclear inflammatory cells. The suppuration that follows is most probably the result of secondary invasion. In the course of degeneration, globular structures con-

taining a fair amount of chromatinic material are formed in the cytoplasm, but they disintegrate as the cell approaches the surface in keratinization. These cytoplasmic inclusions may be analogous to Guarnieri bodies of variola-vaccinia, but are comparatively less stable. They do not seem to develop by enlargement from smaller bodies as do Guarnieri and Bollinger bodies. The swelling of the nucleolus and the formation of a halo of clear space around it resembles a similar change described in varicella (Tyzzer, 1905–6; Rivers, 1926) and variola (Findlay, 1938).

The reticular change in the epidermis proceeds from the surface to the deeper layers. This is in contrast to what happens in variola (Benda, 1927; Michelson and Ikeda, 1927) and bird poxes (Fröhlich, 1934) where the first changes are seen in the basal layer and proceed outwards from there. The vacuolation remains restricted to individual cells and the cell boundaries do not break to form a large vesicle as in vaccinia or foot-and-mouth disease. The C.P.D. vesicle is multilocular and cannot be emptied by a pin prick, while the 'balloon' degeneration seen in some other pox diseases is not observed in C.P.D. In this connection, it may be stated that the opinion of some previous workers that ballooning occurred in C.P.D. is not supported by their own descriptions or illustrations. The published descriptions when compared with Unna's comparative table of reticular and balloon degenerations leave little doubt that only reticular changes have been observed ('alterations cavitaire' of French writers).

The question of the pathological classification of C.P.D. is an important one. Aynaud (1923), Glover (1928) and Bubberman and Kraneveld (1931) considered this disease to be closely related to the vaccinia-variola-alastrim group on the basis of the epidermal degenerative changes even though they were unable to find any cytoplasmic inclusions. It may be pointed out that the reticular (or 'balloon') degeneration is not exclusive to the vaccinia-variola-alastrim group as it also occurs in foot-and-mouth disease and eczema (Hieronymi, 1924, 1927). Saliyi's (1939) finding of cytoplasmic inclusions in cells undergoing this degeneration, however, provided a closer parallel with pox diseases. His work has been confirmed in the present inquiry and a more detailed examination of the nuclear and cytoplasmic changes has provided further evidence of similarity with the classical pox diseases.

CONCLUSIONS

The skin response of sheep to C.P.D. virus consists of a focal proliferation, reticular degeneration and necrosis of epidermal cells and inflammatory reaction in the corium.

In the course of reticular degeneration, globular or subglobular inclusions, measuring 4 to 8 μ in diameter, appear in the cytoplasm of epidermal cells. These inclusions contain some chromatinic material and disintegrate as the cell approaches the skin surface.

The nucleolus is enlarged and frequently surrounded by a clear halo. These changes are similar to those seen in other diseases of the pox group.

ACKNOWLEDGMENTS

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Fig. 2

Keratinization in normal epidermis of sheep. Camera lucida drawings of representative cells.

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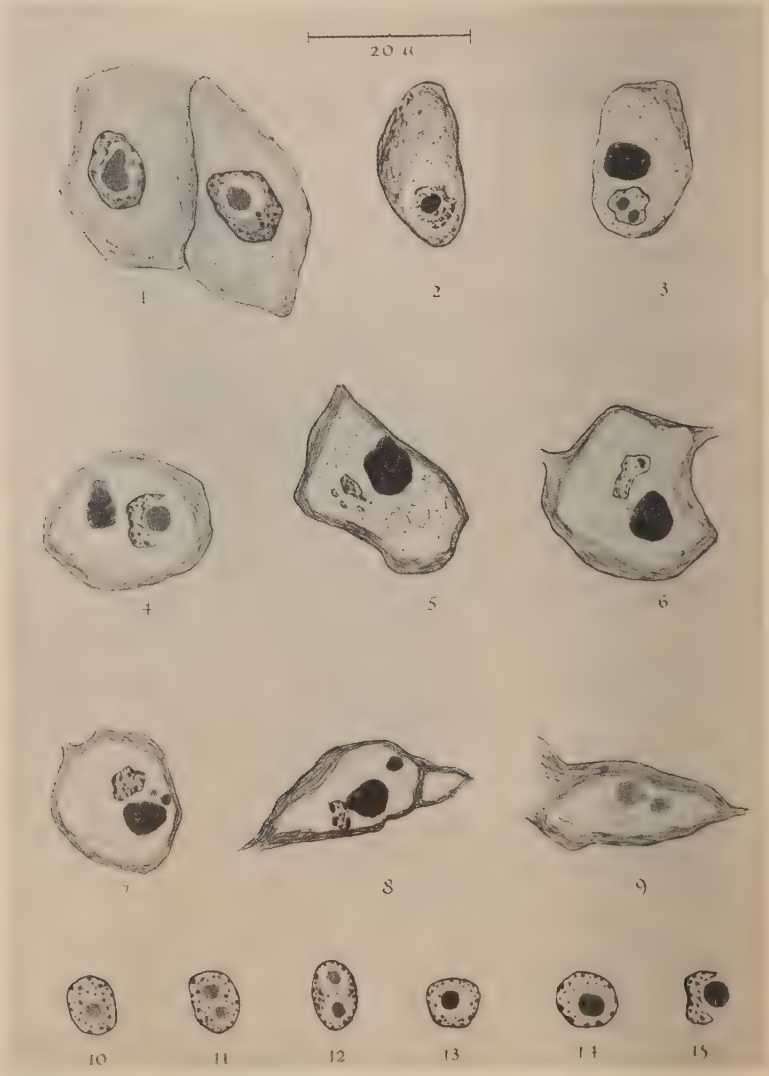
Fig. 5.



Cellular changes in the epidermis. 1–9, progressive changes leading to the migration of tonofibrils towards the periphery and formation of inclusion body followed by its disintegration; 10–11, normal nuclei; 12–15, degeneration of nuclei of affected cells.

Camera lucida drawings of typical cells in a 5-day lesion.

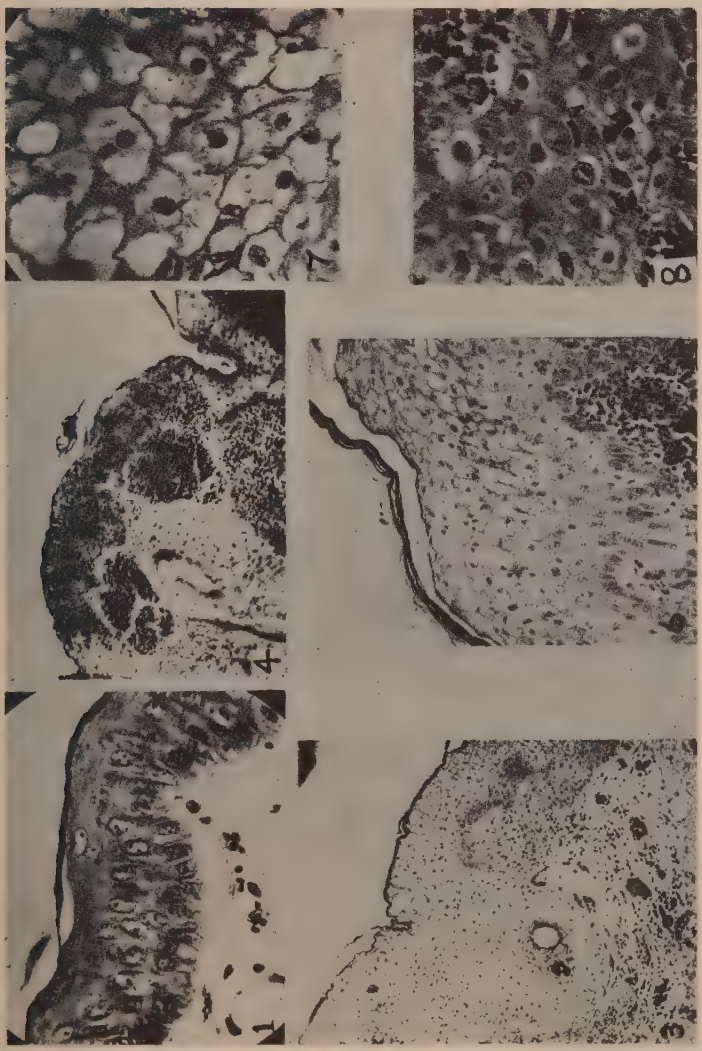


Fig. 1. Normal skin of the inside of thigh of sheep. Iron-alum haematoxylin. × 400.

Fig. 3. Three-day lesion of C.P.D. on skin of lamb. Proliferation and reticular degeneration of epidermis. Inflammatory reaction in the dermis. H. and E. × 75.

Fig. 4. Five-day lesion. Commencing suppuration. H. and E. × 72.

Fig. 6. Four-day lesion showing cytoplasmic inclusions in epidermal cells. H. and E. × 150.

Fig. 7. Epidermal cells in a 4-day lesion showing reticular degeneration and cytoplasmic inclusions. H. and E. × 400.

Fig. 8. Epidermal cells in a 6-day lesion showing enlargement of nucleoli, some of which are surrounded by clear halos. Iron-alum haematoxylin. × 400.

UTERINE RESPONSE TO ADRENALINE IN INTACT COWS AND SHEEP

By

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INTRODUCTION

The response of the myometrium to adrenaline differs widely between species, and within species at different stages of reproductive activity. In ruminants this response has been described in contradictory terms. However the authors concerned have almost invariably confined their studies to in vitro methods, which are a very unreliable index of uterine responses in intact animals (Robson and Schild, 1938a; Garrett, 1954), and generalisations in some cases have been based on narrowly conceived experiments (Gunn, 1944).

In cattle, Thienes (1928) and Alexander (1945) reported an inhibitory response of the uterus to adrenaline, in pregnant and non-pregnant cows. On the other hand Cupps and Asdell (1944) and Asdell, de Alba and Roberts (1945) obtained both contractile and inhibitory responses of the non-pregnant bovine uterus. Sommer (1932) described contraction as the typical response of the pregnant uterus to adrenaline.

In small ruminants inhibition of the pregnant uterus induced by adrenalin has been reported in the sheep (Gunn, 1944; Alexander, 1945) and goat (Newton, 1934; Gunn, 1944). However, Ambache and Hammond (1949) working with pregnant sheep, recorded both contractile and inhibitory responses. In the non-pregnant ewe both stimulation (Gunn, 1944) and inhibition (Alexander, 1945) of the uterus have been described.

The present investigation extends observation of this phenomenon to the living animal, at different stages of the ovarian cycle in cows, and during pregnancy and at parturition in both cows and sheep. The results indicate that in the past too rigid an attempt has been made to classify responses as either inhibition or stimulation and to associate either of these responses with pregnancy *per se* rather than with variations in sex hormone secretion.

METHODS

Administration of drugs. Adrenaline hydrochloride solution B.P. (Parke Davis & Co.) was given intravenously to cows and to ewes, in various stages of reproductive activity as indicated in Tables 1 to 5. In cows it was possible to compare in the same individual, the response of the pregnant and non-pregnant uterus to the same dose of adrenaline.

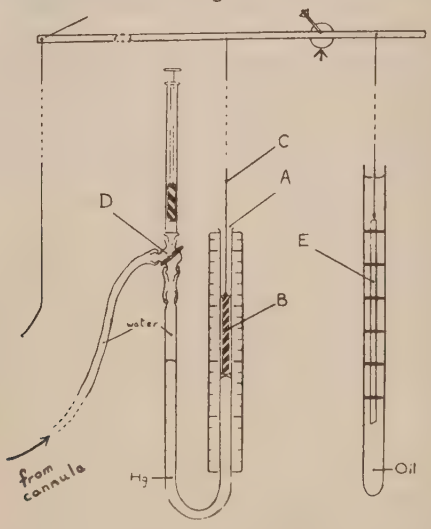
For repetitive intravenous administration of adrenaline and other drugs simple venepuncture was impracticable, each puncture eliciting a general response of the whole animal which would have interfered with

the recordings. Instead an indwelling polythene catheter (Bore 0·8mm. Length 4 feet) was inserted 12″ to 20″ into an external jugular vein, and the free (external) end connected to the outlet of a small stainless steel 3-way tap of very small dead space. One arm of this tap was connected to a transfusion bottle from which a slow drip of saline was maintained throughout each experiment. Into the remaining arm of the tap drugs were injected when necessary, using an insulin syringe graduated to 0·01 ml. This system enables drugs to be given intravenously in accurate doses, at predetermined intervals, without disturbing the animal.

Recording apparatus. The method used was similar in principle to that described by Schild, Fitzpatrick and Nixon (1951) for recording simultaneously the contraction of both cervix and corpus uteri in man. In the present investigation cervical contractions were also recorded but this aspect will be dealt with in a separate publication.

Intra-uterine pressure is recorded from a small latex rubber balloon (capacity 2 ml.) mounted terminally on a hollow metal cannula. A few inches from the tip of the cannula a second smaller balloon is situated. The cannula is double, consisting of two co-axial channels each of which communicates directly with the lumen of one of the balloons and peripherally, via pressure tubing, with a mercury manometer. The whole system is filled with water. Pressures to which the balloons are subjected are transmitted to the manometers, the movements of which are linearly magnified and recorded by the method indicated in Fig. 1. In the open

Fig. 1



Recording manometers.
One arm of the mercury manometer is open to the atmosphere (at A) and the other is connected to a three-way tap (D) and thence to the filling syringe, or to the cannula. The mercury in the open arm carries a cylindrical float (B) which is connected by a thread (C) to a lever writing frontally on a kymograph. The lever carries a counter weight (E) the movements of which are oil-damped. The moment of counterweight (E) about the fulcrum is less than that of the float (B) so that the latter always follows the movement of the mercury level.

arm of each manometer a cylindrical float which rests in contact with the surface of the mercury is connected by a thread to a lever writing frontally on a kymograph. A counterweight of lesser moment, is attached to the lever on the opposite side of the fulcrum, its movements being oil-damped to eliminate irrelevant rapid movements of the lever, such as those due to general body movement.

After sterilization, and with the balloons completely deflated, the instrument is introduced per vaginam, to the external cervical os, and then advanced, guided by rectal examination, until the tip lies in the uterus approximately at the point of bifurcation of the horns. The second balloon then lies mid-way along the cervical canal. This position is maintained by clipping the shaft of the cannula to the vaginal protruberance of the cervix. The distance between the two balloons varied with different cannulae but was not less than 10 and not more than 14 cm. The external diameters were 6·0 mm. for use in sheep and 8·0 mm. for cows.

When in position the balloons are partially inflated by injecting into each water system a volume of water slightly less than the mould volume of the balloon. With the balloons only partially inflated the system records fall as well as rise in pressure. In pregnant animals some difficulty is found in introducing such cannulae owing to the accumulation of a tenacious mucus in the cervical canal. The manipulation is greatly facilitated by preliminary catheterisation with a conventional clinical uterine catheter.

The procedure did not necessarily result in abortion. Six cows were used, in five of which repeated cannulations were carried out at intervals ranging from a few days to 9 weeks. Two cows aborted (Nos. 22 and 18) and of these one, which was used only once, was found to be heavily infected with *Br. abortus*.

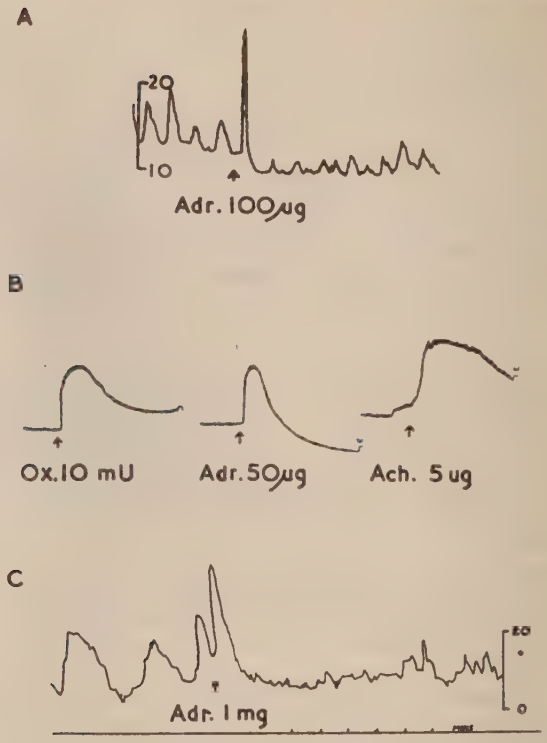
For the cannulation and throughout the subsequent experimental period, all cows were maintained in the normal standing posture under posterior epidural anaesthesia using approximately 6 ml. of a 2·5 per cent solution of procaine hydrochloride.

In sheep all experiments were performed under general anaesthesia using sodium pentobarbitone or cyclopropane. In order to catheterise per vaginam it was found necessary to support the cervical canal with a hand within the pelvis. A preliminary laparotomy was thus required in this species: the laparotomy incision was closed before recording uterine activity. Each sheep was studied only once during pregnancy.

RESULTS

In most experiments the response of the uterine muscle consisted of a short stimulation followed immediately by a longer inhibition as illustrated in Figs. 2a and 2c. The diphasic nature of the response could also be detected in vitro as shown in Fig. 2b. It is proposed to accept this as the basic pattern of response in these species. The intensity of the contractile and inhibitory components varied with the stage of reproductive activity and this variation will be described separately for non-pregnant cows, pregnant cows and pregnant sheep.

Fig. 2



Diphasic responses of uterine muscle to adrenaline (Adr.) showing initial contraction and subsequent inhibition.
A: Pregnant sheep uterus *in vivo*.
B: Non-pregnant bovine uterus *in vitro*.
C: Non-pregnant bovine uterus *in vivo*.
Note that the response of the isolated strip (B) to adrenaline shows a phase of secondary inhibition which is absent from the response of the same strip to oxytocin (ox) and acetylcholine (Ach.)

Cattle

In order to assess quantitatively the variation in the contractile and the inhibitory components of uterine response, the following criteria are adopted. Contraction is expressed as the maximum increase in pressure, over and above that of previous spontaneous contractions. Inhibition is expressed not as the magnitude of the fall in pressure — the pressure frequently fell to zero—but in terms of the duration of the inhibition, measured in minutes.

Non-pregnant cows. Uterine responses to adrenaline in 19 experiments during the ovarian cycle are summarized in Table 1. In Fig. 3 the mean contractile and mean inhibitory effects of adrenaline

are plotted in relation to the day of the ovarian cycle. It can be seen that during the luteal phase the responses were predominantly motor. The magnitude of contraction at this stage was greater than at any other stage and the inhibitory component was short or absent. A typical kymograph tracing is shown in Fig. 4a. During the late luteal phase and pro-oestrus, the magnitude of contraction diminished whilst the duration of inhibition increased. At oestrus the inhibitory component was still more pronounced and the contractile response further reduced or abolished. Clear inhibition without a contractile component was seen shortly after oestrus (Fig. 4b).

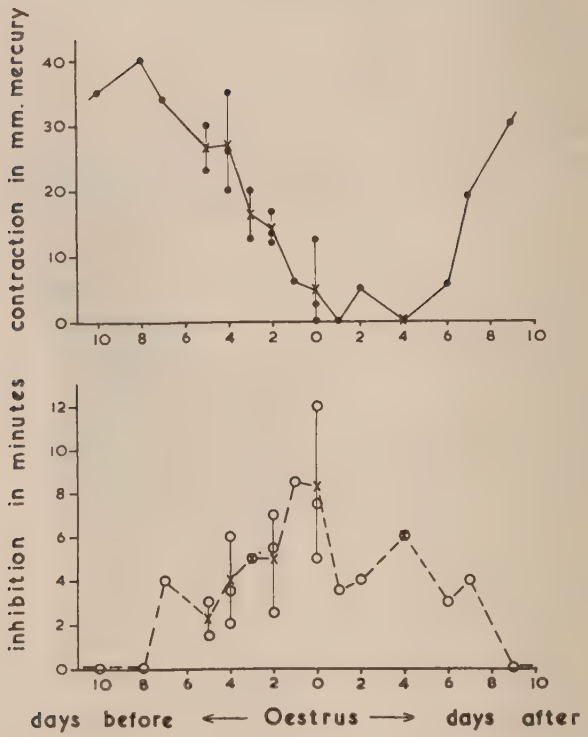
TABLE 1

THE EFFECT OF ADRENALINE ON THE UTERUS OF THE NON-PREGNANT BOVINE

No. of animal	*Stage relative to oestrus*	*Dose of Adrenaline (micrograms)*	*Increase in pressure (mm. Hg.)*	*Duration of inhibition (minutes)*
2	12 d. prior	250	30	0
68	10 d. prior	200	35	0
16	8 d. prior	300	40	0
22	7 d. prior	250	34	4
25	5 d. prior	200	30	1½
23	5 d. prior	250	23	3
22	4 d. prior	250	26	3½
22	4 d. prior	500	35	2
22	4 d. prior	1000	20	6
16	3 d. prior	250	20	5
16	3 d. prior	200	12½	5
23	2 d. prior	200	12	5½
23	2 d. prior	1000	17	2½
22	2 d. prior	150	13½	7
22	1 d. prior	250	6	8½
22	oestrus	500	2½	12
16	oestrus	250	0	5
68	oestrus	250	12	7½
68	1 d. post	100	0	3½
23	2 d. post	250	5	4
23	4 d. post	350	0	6
23	4 d. post	200	0	6
68	6 d. post	250	5½	3
24	7 d. post	250	19	4

In four experiments the uterine response to adrenaline was recorded before and after the intramuscular injection of stilboestrol dipropionate in oil. The animals were initially in anoestrus of long duration. In three cases single doses of 20 to 35 mg. were given: in the fourth an initial dose of 30 mg. was followed 48 hours later by another 20 mg. These doses are greater than those required to elicit oestrus behaviour in spayed heifers (Asdell, 1946; de Alba and Asdell, 1946) and in our animals produced changes in mating behaviour, in the nature of cervical secretion, and in uterine motility, which are typical of natural oestrus.

Fig. 3



The magnitude of the contractile component (above) and the inhibitory component (below) of the response of the bovine uterus to adrenaline is plotted against the stage of ovarian cycle. Circles represent individual observations. Crosses represent means.

The responses are summarized in Table 2 adopting the same criteria as previously. All four experiments agree in showing that stilboestrol favoured an inhibitory response to adrenaline, the magnitude of which increased over a period of several days, as illustrated graphically in Fig. 5. The degree of inhibition after stilboestrol was greater than that seen during the normal cycle, probably due to the large doses of synthetic oestrogen used. The contractile component was diminished but not in all cases completely eliminated.

Pregnant cows. Experiments were made during the second half of pregnancy in six cows, five of which were also studied when non-pregnant. Recordings were taken on more than one occasion during pregnancy from four of these animals. Unfortunately it was not possible to make observations at regular intervals throughout pregnancy.

In this group all responses are classified as contraction or inhibition, and no diphasic responses comparable to those in non-pregnant cows were obtained (Table 3 and Fig. 6). [In two experiments (three observations) no response was obtained and this requires further comment in that an inhibitory effect may have escaped registration. During pregnancy the uterus is relatively quiescent: in these two animals no spontaneous activity was recorded and the intrauterine pressure was negligable. Under these circumstances inhibition of the uterine muscle would not be registered, although stimulation would be obvious. However, when rhythmic uterine contractions were induced artificially with oxytocin, a further dose of adrenaline elicited an inhibitory response without a contractile component. It is probable that in these experiments showing no response, the effect of adrenaline would have been to inhibit spontaneous activity had this been present.]

TABLE 2

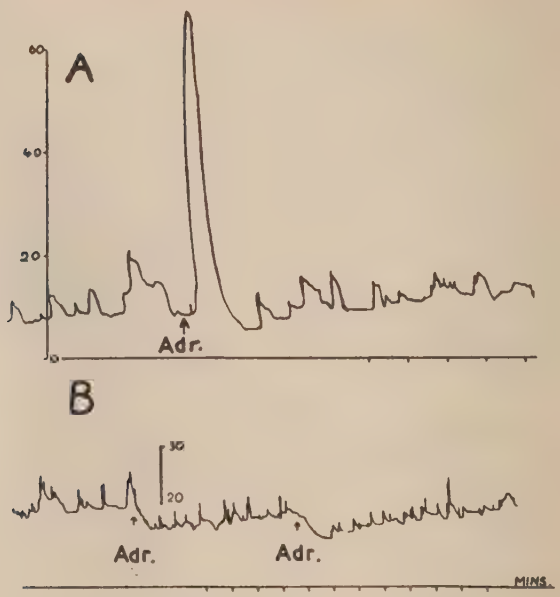
THE EFFECT OF STILBOESTROL DIPROPIONATE ON THE ADRENALINE INDUCED RESPONSE OF THE BOVINE UTERUS

Dose of Stilboestrol	*Duration of action (days)*	*Dose of Adrenaline (micrograms)*	*Increase in pressure (m.m. Hg.)*	*Duration of inhibition (minutes)*
nil	—	250	40	0
50 mg*	1	250	30	0
	3	200	13	3
	6	100	12	13½
	7	250	11	13
nil	—	250	19	3½
35 mg.	3	250	0	4
	3	800	6½	9
nil	—	250	22	2
20 mg.	7	250	0	7
nil	—	500	13	0
20 mg.	3	500	1·5	5
	5	500	0	10½

*In this animal an initial dose of 30 mg. was followed 48 hrs. later by another of 20 mg.

Over the group as a whole our results indicate that during the second half of pregnancy, the corpus uteri of the cow has lost the contractile component of response to adrenaline. Only two experiments showed a contractile response, one being at the 19th week of pregnancy and the other one week prior to parturition. These exceptional responses will be discussed below. This characteristic inhibitory response is in marked contrast to the pure contractile response seen in the luteal phase in the non-pregnant cow and this contrast is particularly significant since the same animals were used, with the same range of doses of adrenaline. Fig. 6 illustrates different

Fig. 4



Responses of the bovine uterus to adrenaline.
A: during the luteal phase.
B: four days post-oestrus.

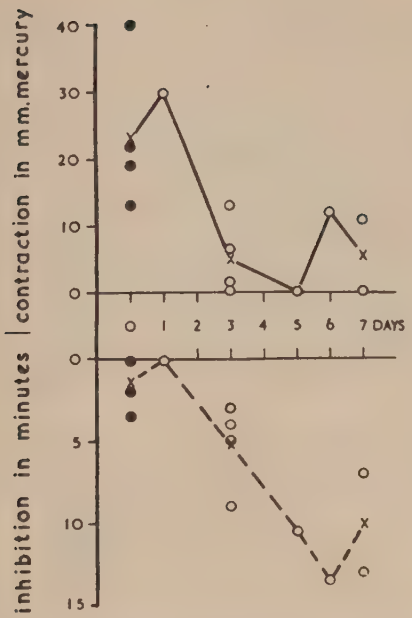
responses recorded from the same cow when pregnant and when non-pregnant.

Post-partum period. The response to adrenaline was studied in the period after parturition and before the re-establishment of cyclic ovarian activity. Eleven experiments were made on 5 cows between the day of parturition and the 51st day post partum. In this group oestrus was first seen in two animals 49 and 51 days after parturition respectively and no experiments were made later than this. During this period responses were predominantly inhibitory as summarized in Table 4 and Fig. 7. From the 2nd to the 13th day post partum relaxation was unaccompanied by any contractile component. Subsequently a slight primary contraction was recognised at the 20th day and the duration of inhibition diminished. These changes progressed until at the 45th day and 4 days before the return of oestrus in this animal a typical diphasic response was recorded.

Pregnant and Puerperal Sheep

Uterine response to adrenaline was recorded in 15 experiments in the second half of pregnancy, each ewe being studied only once. Three observations were also made in the first five days after par-

Fig. 5



The contractile and the inhibitory components of the uterine response to adrenaline in the cow are separately plotted against the interval (in days) following the injection of stilboestrol dipropionate in oil.
Circles represent individual observations; Crosses represent means for each day; Closed circles represent pre-treatment responses; Open circles represent responses after administration of oestrogen.

turition. The character of the response to adrenaline follows a regular pattern related to the stage of pregnancy as summarized in Table 5.

Our earliest experiments were made at the 11th week of pregnancy. In this, and in the three subsequent experiments, contractile components were present, the responses being either pure contraction or diphasic. Between the 15th week and parturition inhibition was almost invariably obtained. Only one contractile response was seen during this period, in a ewe at full term. This appeared to anticipate a change of response to the contractile form at the end of pregnancy, since the contractile component of response was also present in all the responses recorded post partum. The sequence of response patterns is indicated diagrammatically in Fig. 8.

DISCUSSION

Our results, particularly in non-pregnant animals, indicate that the two components of response, inhibition and contraction, are not mutually exclusive as appears to have been assumed by many

previous authors. Diphasic responses are the rule in the non-pregnant cow, with brief initial stimulation preceding a long-lasting inhibition. Similar diphasic responses have also been obtained in these species with several close chemical homologues of adrenaline (unpublished observations). The stage of inhibition cannot be attributed to an indirect circulatory effect of adrenaline, such as production of uterine ischaemia, as has been suggested by Schofield (1952) for the rabbit, since similar responses are seen in vitro. Further, Robson and Schild (1938b) have shown in the intact cat that the inhibitory or contractile nature of uterine response to adrenaline and other drugs is independent of uterine vasoconstriction. Neither can inhibition be considered as merely secondary to a primary phase of contraction since it can occur without preceding stimulation. Both contraction and relaxation appear to be direct and independent pharmacological responses to adrenaline and recall the known opposing components of the responses of other body systems to this drug.

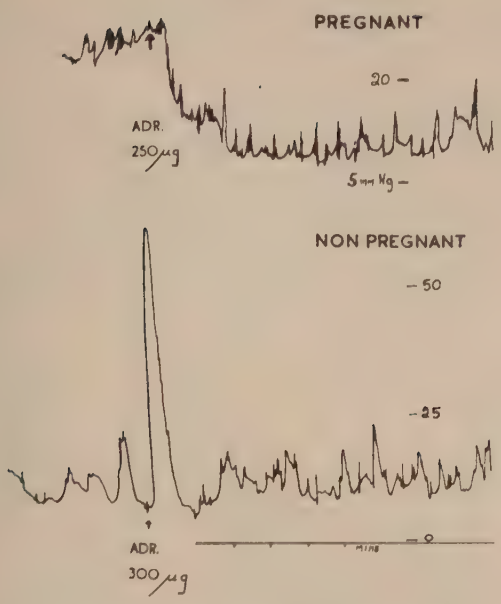
TABLE 3

EFFECT OF ADRENALINE ON BOVINE UTERUS IN PREGNANCY

No. of animal	*Stage of pregnancy*	*Dose of Adrenaline (micrograms)*	*Response*
20	19 weeks	400	contraction
68	21 ,,	250	inhibition
68	21 ,,	800	inhibition
68	24 ,,	250	nil
68	24 ,,	800	nil
20	28 ,,	500	inhibition
20	28 ,,	500	inhibition
22	30 ,,	500	inhibition
18	30 ,,	500	nil
16	38 ,,	250	inhibition
16	38 ,,	450	inhibition
23	39 ,,	250	contraction
23	39 ,,	500	contraction
16	39 ,, 2 days	500	inhibition
16	39 ,, 4 ,,	250	inhibition
23	39 ,, 5 ,,	500	inhibition
16	39 ,, 6 ,,	100	inhibition

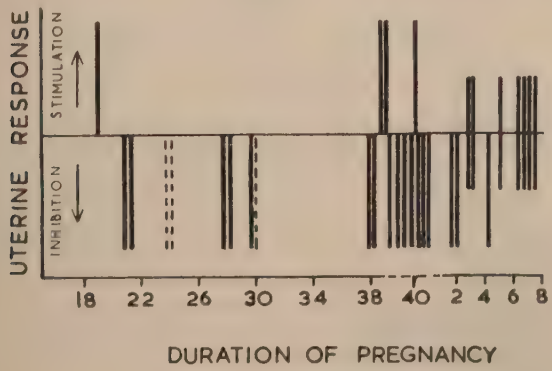
In the past many investigators of uterine response to adrenaline and to stimulation of the hypogastric nerve in laboratory animals have failed to recognise diphasic responses in their own right and instead have adhered to a classification recognising only stimulation or inhibition (Gruber, 1933; Lands, 1949; Reynolds, 1949). This appears to be due to uncritical generalisations based on the predominant responses reported by the early investigators in this field (Langley and Anderson, 1895; Langley, 1901; Cushny, 1906; Dale, 1906; Barger and Dale, 1910). Diphasic responses were in fact described by these earlier authors, albeit for a minority of experiments, in the rabbit, cat and dog. Recently however there has been

Fig. 6



The uterine responses to adrenaline during pregnancy and when non-pregnant are compared in the same individual animal. In pregnancy the contractile component is lost.

Fig. 7



The nature of the uterine response to adrenaline in the cow is presented diagrammatically in relation to the duration of pregnancy in weeks. Pure contractile response is shown wholly above the horizontal axis, pure relaxation wholly below, and diphasic response is intermediate. The dotted lines represent experiments which indicated only that contractile response was certainly absent.

fresh recognition of the occurrence of such diphasic responses in these species (West, 1949; Greeff and Holtz, 1951; Schofield, 1952) and in man (Brown and Wilder, 1943; Woodbury and Abreu, 1944).

TABLE 4

THE RESPONSE OF THE BOVINE UTERUS TO ADRENALINE AFTER PARTURITION

No. of animal	*Post-partum period (days)*	*Dose of Adrenaline (micrograms)*	*Response*
18	0	500	stimulation
16	1	250	inhibition
23	2	500	inhibition
68	3	250	inhibition
22	13	500	inhibition
22	13	1000	inhibition
22	20	250	diphasic
22	20	500	diphasic
23	30	500	inhibition
23	36	250	diphasic
22	45	200	diphasic
22	45	1000	diphasic
22	49 (oestrus)	500	diphasic
68	51 (oestrus)	250	diphasic

TABLE 5

ACTION OF 100 MICROGRAMS ADRENALINE (i.v.) ON THE UTERUS OF THE SHEEP DURING PREGNANCY AND THE PUERPERIUM

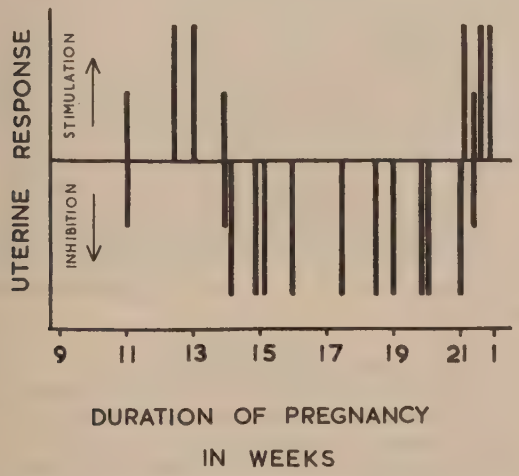
Stage of pregnancy	*Predominant response*
11 weeks	diphasic
12½ weeks	stimulation
13 weeks	stimulation
14 weeks	diphasic
14 weeks	inhibition
15 weeks	inhibition
15 weeks	inhibition
16 weeks	inhibition
17½ weeks	inhibition
18½ weeks	inhibition
19 weeks	inhibition
20 weeks	inhibition
20 weeks	inhibition
21 weeks	stimulation
21 weeks	inhibition
3 days post-partum	diphasic
4 days post-partum	stimulation
5 days post-partum	stimulation

Our view of the normal occurrence of diphasic uterine responses to adrenaline in ruminants is supported by other published work. Thus the figures of Alexander (1945) illustrating 'inhibitory' response of the bovine uterus to adrenaline may be interpreted as including a preliminary contraction. Ambache and Hammond (1949) also

mention after-inhibition following a motor response of the sheep uterus to adrenaline. Finally, in work published after our investigations were begun, Hays and VanDemark (1953) clearly describe diphasic response of the uterus of the intact non-pregnant cow.

Our results indicate that in the intact non-pregnant cow the relative preponderance of the two components, which together make up the diphasic uterine response to adrenaline, is influenced by variation in sex hormone secretion during the ovarian cycle, oestrogen re-enforcing inhibition and progesterone contraction. This is amplified by our experiments in which stilboestrol treatment favoured inhibitory response. These findings agree with the observations made in vitro by Cupps and Asdell (1944) and Asdell *et al* (1945).

Fig. 8



The nature of the uterine response to adrenaline is plotted diagrammatically against duration of pregnancy in weeks for sheep. In each case pure contractile response is shown wholly above the horizontal axis, pure relaxation is wholly below, and diphasic response is intermediate.

In pregnant animals our results are consistent in cows and sheep. Furthermore the same sequence of response patterns is also given by the cervix uteri in both species (unpublished observations). In the sheep our earliest observations, at about the middle of pregnancy, were of contractile or diphasic responses. At all later stages of pregnancy until full term, only purely inhibitory responses were recorded in response to a standard dose of adrenaline. At the end of pregnancy there was a return of the contractile component which persisted for several days post-partum. In the cow, corresponding contractile response at mid-pregnancy was possibly represented by a single observation at 19 weeks — the earliest stage available to us. Apart from this, inhibitory responses were obtained in the second half of

pregnancy with doses of adrenaline which produced contraction in the same animals when non-pregnant. It would seem, therefore, that sufficient oestrogen is secreted during this stage of pregnancy to affect corpus response, despite the coincident secretion of progesterone. At term and at parturition, diphasic and contractile responses were obtained in cows, as in sheep, suggesting considerable variation in the pattern of hormone secretion during this period. During the puerperium there was some difference between the results in the two species. In the sheep, diphasic and contractile responses were recorded up to the fifth day post-partum, whereas in the cow a purely inhibitory pattern was recorded 24 hours after delivery and repeatedly thereafter. Presumably this is a manifestation of differences in sex hormone secretion, and suggests a relative oestrogen dominance in the cow, possibly related to the special requirements of lactation in this species.

Contractile uterine response (in vitro) to adrenaline in early pregnancy, succeeded by inhibitory response in the later stages of pregnancy was also noted in the sheep by Ambache and Hammond (1949) who are thus in agreement with us. The consistently inhibitory responses of the pregnant uterus of the goat and the sheep described by Newton (1934) and by Gunn (1944) now fall into agreement, in that their animals were all either in the last third of pregnancy (Newton) or all at term (Gunn). Sommer (1932) describing contractile response of the pregnant bovine uterus, also recognised a reversal to inhibition which he claimed was associated with degeneration of the corpus luteum.

Clearly, statements describing the reactivity of the pregnant uterus in any species should be qualified by reference to the stage of pregnancy. In this connection it is interesting to reflect that in the cat uterine contraction to adrenaline, long accepted as being the typical response during pregnancy, was originally so described by Dale (1906) for *early* pregnancy. The possibility of a different response at the later stages of pregnancy in this species had not received attention until West (1949) reported a change from the contractile response in early pregnancy to an inhibitory response in late pregnancy. A similar trend to inhibitory responses to adrenaline in the later stages of pregnancy has been observed in vivo in man by Alvarez and Caldeyro (1952). In the rabbit, which has long been regarded as consistently showing contractile response to adrenaline, there is evidence that prolonged luteal activity is associated with loss of contractile response and on occasion its replacement by inhibition (Sauer, Jett-Jackson and Reynolds, 1935).

It is perhaps no longer relevant to speak of qualitative species differences in uterine response to adrenaline during pregnancy. It is possible that there may be a pattern of response during pregnancy common to all species, namely contraction in early pregnancy and relaxation in late pregnancy, and that differences between species are matters of degree and not principle. Certainly it appears no

longer useful to develop a classification in which uterine response during pregnancy in various species is described as either contraction or relaxation.

CONCLUSIONS

The response of the myometrium to adrenaline, given intravenously, has been studied in non-pregnant cows and in the second half of pregnancy in cows and sheep. The characteristic response is diphasic, a short contraction being followed by a longer inhibition.

In the non-pregnant cow luteal activity is associated with a predominantly contractile response. Oestrogen dominance is associated with a predominantly inhibitory response. The response changes qualitatively during pregnancy in both species. At mid-pregnancy a contractile component may be recognised but for most of the second half of pregnancy the response is inhibitory. About the time of parturition contractile responses reappear.

These results are discussed in relation to previous work in these and other species. The validity of claims that the nature of myometrial response to adrenaline differs fundamentally between species is disputed.

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ROUND HEART DISEASE IN POULTRY

By

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INTRODUCTION

In an earlier paper, Wilson (1948) described Round Heart Disease in Scotland and discussed the possibility of a hereditary basis. In a later paper (Wilson and Siller, 1954) the position was reviewed in the light of further experience. The increased and well marked seasonal incidence of the disease was noted, its pathology described and aetiology discussed. The history of outbreaks varied widely. In most there were indications of genetic predisposition, in others birds were attacked apparently indiscriminately. The present paper reviews the current position of round heart disease in Scotland and the 6 northern counties of England, the area served by this laboratory, and describes further attempts to reproduce the disease under laboratory and farm conditions.

Round heart disease appears to be becoming more common in other parts of Great Britain, but the increase does not seem to be as marked as in the area under review. It is a well recognised condition in Europe (Wilson and Siller, 1954), but from the discussion of the paper by Wilson (1953) it was evident that it was unknown in North America. Bankier (1954) described outbreaks in 3 widely separated flocks in British Columbia in 1953 and considers this to be the first record of the occurrence of round heart disease in Canada or possibly in North America. He also recorded a single outbreak in 1956 (Bankier, 1956). Round heart disease has also been described in Canada by Bigland (1955) who observed the disease in 6 flocks in Alberta in 1954. Several outbreaks have occurred during the past 2 years (Bigland, 1956). So far as the writer is aware there are no published accounts of the occurrence of the disease in the U.S.A., but it has been seen in a single flock in New York State in 1955 and 1956 (Levine, 1956).

RESULTS

Epidemiology

Incidence. Through the co-operation of the field staff of the Animal Health Division it was possible to arrange a field investigation into many of the outbreaks and a number were visited by the writer. The chief points of enquiry were the breed or breeds affected, evidence of particular selectivity or localisation of the disease or association with a particular system of husbandry and evidence of spread by contact or other means or connection with a known outbreak. It will be seen (Table 1) that apart from 1955 there has been a marked annual increase in the outbreaks of round heart disease that have come to our notice and that during the first 6 months of 1956 the rise was spectacular.

TABLE 1

SUMMARY OF OUTBREAKS OF ROUND HEART DISEASE (JANUARY 1950 TO JUNE 1956)

Year	*Type of husbandry*					*Breeds affected*					*Number of groups affected in outbreak*		
	Number of outbreaks	*Free range*	*Intensive (Deep litter)*	*Semi-intensive*	*Not specified*	*Leghorn*	*R.I.R.*	*Other breeds*	*Crosses*	*Not specified*	*Single*	*2 or more*	*No history*
1950	8	1	1		6	1	3		1	3	1		7
1951	11	1	1		9	6	2		1	2	2		9
1952	19	3	9	1	6	5	7	1	7		6	4	9
1953	39	5	28	3	3	7	10		20	2	26	3	10
1954	58	3	42	2	11	22	11	1	21	3	34	5	19
1955	49	11	28	1	9	16	14		18	1	37	1	11
1956*	93	7	65	4	17	39	10		39	5	47	8	38
Total	277	31	174	11	61	96	57	2	107	16	153	21	103

*First 6 months only

Breeds affected. The disease occurs most often in all types of Leghorn viz. Black, Brown, White and Exchequer and in crosses (Table 1). It is of interest that the cases described by Bankier (1954) and Bigland (1955) were also in White Leghorns. The disease is also common in the Rhode Island Red. Single outbreaks occurred in New Hampshire Reds, Anconas and Barred Rocks, but the disease was not seen in Light Sussex, Buff Rocks or White Wyandottes. In an outbreak at a Poultry Production and Progeny Trial recorded by Gordon (1952) the disease caused nearly 60 per cent of the mortality in the White Leghorn section and approximately 30 per cent in the Rhode Island Red section. No case was recorded in Light Sussex birds.

Age incidence. In almost all outbreaks pullets were involved and the youngest birds to be affected were just over 8 weeks old. The disease was seldom seen in birds over 1 year old. In one outbreak however, there was a history of birds almost 2 years old being involved, but as the birds were unrung and in a mixed group of pullets of the same breed there is some doubt as to their age.

Environment. Attention has already been drawn to the increased number of outbreaks in fowls kept on deep litter (Wilson and Siller, 1954). It was suggested that this observation might have no real significance since the greater incidence coincided with a period of increased popularity of the deep litter system. It now seems however, that the disease may have a definite connection with this particular system (Table 1). Of 216 affected flocks in which knowledge of the type of husbandry was available, 174 were on built-up litter, 31 on free range and 11 were kept semi-intensively. No outbreaks occurred in laying batteries.

In many outbreaks there was a history that the litter was damp, usually the result of insufficient ventilation or inadequate insulation of the building. It is of interest that investigation of one of the most serious outbreaks showed the disease to be confined to one of 2 almost identical adjacent deep litter houses. The 300 Leghorn cross pullets were from a single source and had been equally distributed at random between the 2 houses; food and litter were from a common supply. There was a marked difference in the condition of the litter in the 2 units. In the house in which 10 to 12 deaths from round heart disease were occurring daily, ventilation was less effective and the litter was damp and cloggy. In the other house in which not a single death occurred from round heart disease there was better ventilation and the litter was dry and "well made." On several farms where birds of the same breed or strain were maintained under different systems of management, usually deep litter and free range, the disease affected those on deep litter only. In practically every outbreak there was a history of grain having been fed by scattering on the litter.

Number of groups affected in outbreaks. In 153 of 174 outbreaks in

which a history was available only a single group of fowls was affected, while in 21 outbreaks the disease affected 2 or more groups (Table 1). These figures probably exaggerate the preponderance of single group outbreaks as the poultry enterprise on a number of farms on which the disease occurred comprised a single unit only, usually a deep litter house.

Seasonal incidence. The seasonal incidence (Table 2) was much the same as was noted formerly, most cases occurring during the first 3 months of the year.

Experimental

In an earlier paper (Wilson and Siller, 1954) the possibility of nutritional aetiology in round heart disease was discussed. In view of the association of Vitamin E and cardiac function in a number of animals, experiments were begun to investigate a possible connection between this vitamin and round heart disease.

A pen of 20 Brown Leghorn chickens 10 weeks old and a pen of 16 Black Leghorn x Rhode Island Red pullets 10 months old received a diet free from Vitamin E consisting of dried skim milk powder, 68 per cent; purified starch, 26 per cent; sawdust, 2 per cent; cod liver oil (solvitax), 4 per cent; with a small amount of mineral mixture added, for $2\frac{1}{2}$ years and 2 years respectively. A single control pen of 16 Black Leghorn x Rhode Island Red pullets, 10 months old and sisters of the birds in the second experimental pen received the same diet with the addition of α tocopherol acetate (Roche) at the rate of 1·0 g. per 100 ml. of cod liver oil. The birds remained in relatively good health considering the monotonous and unnatural diet they received, but ultimately those in both experimental groups developed a peculiar goose-step-like gait and spent lengthy periods resting in a typical crouching position in which they sank back on to their hocks. No cases of round heart disease occurred. The birds were later mated with cockerels receiving a normal diet. In the experimental groups fertility and hatchability fell rapidly to zero while apparently normal healthy chicks were hatched regularly from the control pen.

The occurrence of round heart disease in poultry apparently associated with the use of zinc feeding utensils was reported by Gerriets (1955). Ensiled steamed potatoes fed at the time of the outbreak contained 240 mg. of zinc per kg.. When the use of zinc vessels was discontinued the disease disappeared. In an attempt to see whether zinc is concerned in the aetiology, 2 groups of 6 Brown Leghorn pullets were fed dry mash containing 250 mg. and 500 mg. respectively of zinc sulphate per kg. continuously for 2 months. All remained healthy and laid well except for one bird in each group which laid only 9 and 3 eggs respectively. During the period of the experiment total egg production of the two groups was 158 and 181 respectively.

TABLE 2

SEASONAL INCIDENCE OF ROUND HEART DISEASE (JANUARY 1950 TO JUNE 1956)

	Jan.	*Feb.*	*March*	*April*	*May*	*June*	*July*	*Aug.*	*Sept.*	*Oct.*	*Nov.*	*Dec.*
1950	3	1	2			1						1
1951	1	3	2	3				1		1		
1952	1	6	4	3					2	1	2	
1953	16	5	4	2	2					3	4	3
1954	10	15	10	1	1	2	2	1		2	9	5
1955	17	7	8	3	3	1	2		1	2	3	2
1956	30	20	21	10	8	4						
Total	78	57	51	22	14	8	4	2	3	9	18	11

Role of Deep Litter.

The higher incidence of round heart disease in fowls kept on built-up litter led to an extensive examination of samples of litter from many outbreaks. Amounts of about 1 cwt. were obtained; a small portion was submitted to bacteriological examination for the presence of pathogenic bacteria and fungi and the remainder was mixed with other samples to establish built-up litter units, in which experimental birds were kept for varying periods of time. Of the micro-organisms isolated only *Aspergillus fumigatus* and possibly *Absidia corymbifera* could be considered likely to be harmful. Once it was established that the disease could be acquired from deep litter from a natural outbreak, the search for a pathogenic agent in the litter was intensified but with no success. Attempts to transmit the disease included keeping fowls on litter from several outbreaks and from a single outbreak, keeping birds in contact with an affected group of fowls in the laboratory and in the field, feeding crude and bacteria-free filtrates of cultures from deep litter to fowls and injecting birds with large amounts of culture or filtrates, feeding and injecting cultures and filtrates of *A. fumigatus* and *A. corymbifera*, and injecting filtrates of heart, liver and intestinal contents of affected birds into fertile eggs, day-old chicks and growing stock.

Keeping fowls on deep litter obtained from outbreaks. Three groups of Brown Leghorn pullets were placed on built-up litter obtained from farms where round heart disease had been diagnosed. The litter was forked over occasionally and kept damp by adding water to it. Six deaths occurred from round heart disease between the 6th and 13th weeks (Table 3).

TABLE 3

EFFECT OF KEEPING FOWLS ON LITTER FROM AFFECTED PREMISES

Pen	*Number of fowls*	*Deaths from round heart disease*	*Time between exposure and death (days)*		
1	25	3	79.	92.	95.
2	25	3	42.	67.	75.
3	24	0		0	

Feeding grain contaminated with deep litter from outbreaks. Grain was mixed with deep litter obtained from outbreaks of round heart disease, soaked in water and retained until it became covered with moulds. This material provided the sole food of 16 Brown Leghorn pullets from October to April, the period in which it was considered round heart disease was most likely to occur. The birds remained in good health and laid reasonably well. No cases of round heart

disease occurred and post-mortem examination of the birds at the end of the period of observation showed no evidence of this disease or of aspergillosis. Similar grain was also fed for 2 months to 8 chickens, 6 weeks of age, without adverse effects.

Filtrates of cultures from deep litter. Samples of deep litter from affected premises were incubated in flasks of nutrient broth pH7·8, (selected to approximate the pH. of deep litter) for 5 days at 37°C. and a crude filtrate prepared by passing the contents through glass wool. By means of a pipette 20 ml. of the filtrate were introduced daily into the crops of 4 birds for 53, 56, 93 and 95 days respectively. In one bird which died the heart was flabby and slightly dilated and there was an accumulation of serous fluid in the pericardial sac. In two of the remaining birds which were killed the hearts were also flabby. The condition, however, appeared to be quite distinct from round heart disease. Crude filtrate was mixed with poultry meal to form a wet mash, and this was fed to 23 Brown Leghorn pullets, daily for 14 days. All the birds remained healthy. Bacteria-free filtrates were prepared from the crude material by passing through Seitz filters and 3 birds received 1 ml. by subcutaneous injection daily for 15 days and 3 birds 2 ml. subcutaneously for 15 days. All remained healthy.

Effect of Aspergillus fumigatus *and* Absidia corymbifera

Large quantities of these fungi were prepared by growing in Sabouraud's broth pH5·5 for 5 days. Whole culture of each fungus in amounts of 60 ml. was instilled into the crops of 4 birds daily for 3 weeks without ill effect and autopsy showed no evidence of round heart disease. Seitz filtrates of the cultures were prepared and 5 birds each received a single dose of 5 ml. intravenously. When killed a month later the heart of one bird was found to be flabby, but the remainder were normal. The effect of repeated dosing was tried by injecting 8 birds with 5 ml. of the filtrates 4 times in 14 days, and 8 birds received 60 ml. of the filtrate orally every day for 3 weeks. All remained healthy and there was no evidence of abnormal hearts at post-mortem examination.

Attempted Transmission by Contact with Affected Fowls

Exposure under natural farm conditions. On 9th February 1956, 40 Brown Leghorn and Rhode Island Red pullets from Lasswade, double wing-banded and leg-banded to ensure positive identification, were placed in a deep litter unit where a serious outbreak of round heart disease was occurring, and 44 Brown Leghorn pullets from the affected group, selected at random, were transferred to Lasswade for simultaneous experimentation under laboratory conditions.

The affected fowls had been hatched in March and April, reared outside and put on deep litter on 1st December. On that day egg production was 12 but by 1st January it had risen to 115 and remained around this figure until 19th January when it started to

fall; on the 26th January it was 66, on 2nd February 55 and 9th February 50. Between 20th January and 9th February, 50 birds died from round heart disease. The deep litter unit consisted of 2 compartments with access through an open doorway. The litter was chaff with lime added and was reasonably dry and in good condition in the inner compartment, but in the outer compartment it was damp and tended to clog. The disease was confined to birds on deep litter; others of the same strain kept on free range and under semi-intensive conditions were unaffected. The birds appeared in fairly good condition; a few had "colds" and others had slightly swollen faces shown later to be associated with Vitamin A deficiency. In one or two birds the comb was cyanosed or shrunken and scaly, consistent with the presence of round heart disease. The outbreak was waning when the birds from Lasswade were introduced but 3 further deaths occurred in the home stock and 2 of the Lasswade birds died with typical lesions of round heart disease. The periods between exposure and death were 24 and 45 days respectively.

Exposure under laboratory conditions. At Lasswade 17 of the 44 imported fowls were placed with 17 Brown Leghorn and Rhode Island Red pullets on built-up litter from the affected farm. During the next 3 weeks there were 4 deaths from round heart disease in the imported birds, but none of the Lasswade birds became affected. Concurrently, 17 imported birds were placed with an equal number of Lasswade pullets on clean deep litter. There was a marked improvement in appearance and general health of the imported birds; no deaths occurred in either group.

The remainder of the imported fowls, 10 in number, were placed in battery cages in close contact with a large number of fowls of the same age and type as those used in the previous experiments. One of the imported fowls died of round heart disease 24 days later. All the surviving birds from the 3 experiments were kept under observation for periods of from 6 to 8 months. At the end of that time they were destroyed but no cases of round heart disease were revealed.

Attempted Transmission by Inoculation of Diseased Organs

Intestinal contents from affected birds were cultured in broth and filtered through glass wool and instilled into the crops of birds for periods varying from 3 to 96 days. Bacteria-free filtrates were injected intramuscularly and subcutaneously into fowls daily in 2 ml. doses for up to 90 days and given orally in 20 ml. doses for up to 94 days. All the birds remained healthy. Fowls injected with emulsions of heart and liver tissue from affected cases failed to develop the disease. The injection of filtrates of diseased organs had no adverse effect on egg embryos or day old chicks.

DISCUSSION

Round heart disease is becoming increasingly important as a cause of loss in poultry as judged by the results of diagnosis at this

laboratory. The occurrence of 93 outbreaks during the first 6 months of 1956 compares with 56 of fowl typhoid, 19 of fowl cholera, 26 of infectious laryngotracheitis and 207 of respiratory diseases excluding the latter disease.

The aetiology is still obscure but the work described confirms the view tentatively expressed previously that there is a definite association between round heart disease and the built-up litter system of poultry management. It has been shown in 2 separate experiments that the disease can be induced in pullets by running them on litter previously used by affected birds, and in a field experiment it occurred in birds placed in contact with both diseased stock and contaminated litter. A similar observation was made by Siegmann and Woernle (1954) who recorded the death from round heart disease of 1 of 6 fowls 47 days after they had been placed in a small pen with a run of 15 square metres with 13 survivors of a group of 26 fowls which had been attacked by round heart disease.

Failure to reproduce the disease by inoculation precludes any definite pronouncement on aetiology, but it would appear that there are two possible interpretations of the results. The disease may be caused either by an infective agent or by some deleterious substance which is present in built-up litter. In the light of the present experimental work the former hypothesis seems the more tenable although the history of most natural outbreaks appears antagonistic to this suggestion, since in only 21 of 174 outbreaks in which the history is known were 2 or more groups of fowls affected. It would appear, therefore, that in addition to the presence of a postulated infective agent other factors play an important role in the expression of the disease; this is by no means uncommon in human and animal infections.

The history of sudden onset of the disease, with high mortality followed in many outbreaks by much less severe symptoms and sometimes by apparent recovery, is not inconsistent with an infectious disease, and in a minority of outbreaks the apparent spread of the disease is characteristic of an infection. The greater prevalence of the disease in birds on deep litter does not detract from the hypothesis of an infective agent as the causal factor, but rather supports it. Once an infection is introduced, the deep litter system of husbandry, efficiently operated or otherwise, provides an ideal means for its build-up. There is a relatively large population within a strictly confined area, droppings accumulate over a lengthy period of time and birds come into more intimate and continual contact with them than in any other system of poultry management. It is also common practice when grain is fed to distribute it on the litter. The greater weight of infection to which birds kept under this system are exposed, assuming an infective agent to be the cause, might explain the absence of the disease in related birds kept on free range. The history that an outbreak often ceases when an affected group of fowls is changed from deep litter to free range

also supports the hypothesis of an infection which becomes diluted when the birds have access to a run. Too much significance cannot be placed on this observation, however, as sudden onset and equally sudden termination are characteristic of round heart disease.

Following the demonstration of intranuclear inclusions which do not appear to have been described by other authors, Wilson and Siller (1954) considered the possibility of virus causation of round heart disease, but the evidence against it seemed overwhelming. Failure of transmission had been recorded by Fischel (1946), Blaxland and Markson (1947), Adsersen (1948), Luke (1948) and Wilson (1948). The inocula used by these workers consisted mainly of suspensions of diseased organs. In the present experiments attention was paid to the possible presence of an infective agent in the excreta of affected birds or in the litter, but it proved impossible to reproduce the disease artificially by any of the means adopted to ensure that large doses of a possible contaminating agent were introduced directly into the bodies of the experimental fowls, both by natural and experimental means. In 3 of 4 birds which received crude filtrates of deep litter daily for from 8 to 14 weeks the hearts were flabby and dilated, but the condition appeared to be quite distinct from round heart disease. All efforts to induce disease by inoculation of material from affected birds failed completely. Siegmann and Woernle (1954) consider that round heart disease may be transmitted by coitus. They produced a condition resembling early lesions of round heart disease in 3 of 4 fowls by injecting into the cloaca a suspension of cloacal mucous membrane from 2 capons which had themselves been similarly injected with cloacal material from 2 cockerels which had died of round heart disease. These findings require further confirmation. The gross and microscopic lesions described were not typical of classical round heart disease as generally recognised and none of the experimental birds died.

The peculiar selectivity of the disease has been stressed. This was apparent in the first field outbreak investigated by the writer in 1942 in which there was a high mortality in White Leghorn pullets, while Rhode Island Red fowls in relatively close contact remained free. In another early outbreak in a pen of mixed pullets, only Leghorns were involved; and in a deep litter house divided by a wire netting screen, a high death rate occurred in one compartment, and not a single death in the other in which birds from a different source were housed. This type of behaviour has continued to be a feature of the disease; in 153 out of 174 outbreaks, for which a history is available, a single group of fowls only has been involved. It seems probable that the occurrence of an outbreak of the disease depends on the degree of susceptibility or resistance possessed by the stock and the weight of infection to which it is exposed at a critical period, generally within the first few months of life. It is obvious that a strong resistance develops with increasing age. This tends to

support rather than invalidate the theory postulated by Wilson (1948) that the disease has a hereditary basis and that some strains of fowl are more liable. The idea of a hereditary anatomical defect has long been discarded. The behaviour of the disease is not unlike that of neurolymphomatosis, in which the incidence in a given strain of fowl varies greatly in different environments and great differences in incidence are shown by different strains in the same environment. The absence of round heart disease in Light Sussex suggests a strong inherited resistance in this particular breed; it may be argued that most birds are possessed of such an immunity, or develop an immunity early in life and that it is the exceptional strain which is susceptible.

The survey has shown that several outbreaks may stem from a single supplier of stock and that the disease may persist for several years or recur intermittently on certain farms. There are records of widely separated outbreaks occurring during the same week in stock from a farm where the disease was known to exist. It has been difficult to assess whether these happenings signify the dissemination of actual infection or indicate a susceptible stock exposed to infection on the new premises.

Many of the arguments in favour of an infective agent apply, at least in part, to the presence of some deleterious or toxic material in the litter as a cause of the disease, but it is difficult to reconcile all the facts with this theory. The possibility that the disease might be an expression of anaphylaxis as a result of birds becoming sensitised to some constituent in the pasture was suggested by Luke (1948), but this aspect does not appear to have been explored further. On several occasions when survivors of an outbreak have been transferred to clean litter at the laboratory, they have shown a striking and almost immediate improvement and birds which were ailing and expected to die have recovered. This has also been noted by Levine (1956). In the present series of experiments there were no further deaths in any of the 17 birds obtained from the field outbreak and put on fresh litter, whereas 4 of the 17 imported fowls kept on litter from the farm died from round heart disease.

The suggestion that the ingestion of zinc may be associated with the disease (Gerriets 1955) may be discounted. In one of the Scottish outbreaks there appeared to be circumstantial evidence that a chemical poisoning was implicated. The appearance of the disease followed the feeding of large quantities of grain artificially sprouted in a chemical medium and ceased when the practice was discontinued, but efforts to reproduce the disease by prolonged feeding of sprouted grain prepared by the same method and by the addition of the chemical mixture to the drinking water in the laboratory were unsuccessful (Wilson, 1952). Work on the disease is being continued mainly along the lines of searching for a virus or other infective agent. One of the difficulties is to obtain suitable experimental stock. It seems probable that survivors of an outbreak

possess some resistance which may be passed to their chicks. An attempt is being made to rear large numbers of chickens from the birds which were kept in battery cages to see whether their subsequent freedom from the disease may have been due to a lack of exposure or insufficient exposure to infection rather than to the possession of any inherent or acquired immunity, and whether chickens bred from them may be susceptible.

CONCLUSIONS

A survey of 216 outbreaks of round heart disease showed that 174 (80 per cent) occurred in birds kept on built-up litter.

The disease occurred in pullets kept on built-up litter from a field outbreak of the disease. The period of time between exposure and death varied from 42 to 95 days.

The disease caused the deaths of 2 out of 40 pullets, 24 and 45 days after they had been placed with fowls in a deep litter house in which the disease was occurring.

Experiments to determine the nature of the causal agent were unsuccessful; the possibility that the disease is infectious is discussed.

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Heart and liver of bird which died from round heart disease 92 days after placing on deep litter from an outbreak. Note rounded and dimpled apex of left ventricle and dark appearance of the liver as a result of terminal venous congestion.

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DIETHYLCARBAMAZINE IN THE TREATMENT OF LUNGWORM INFESTATION OF CALVES DUE TO *DICTYOCAULUS VIVIPARUS*

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INTRODUCTION

Diethylcarbamazine acid citrate (1—diethylcarbamoyl : 4—methylpiperazine dihydrogen citrate—British Veterinary Codex 1953) was developed following the last war in a search for drugs to combat filariasis in troops in the Far East. The literature on its use in man has been well reviewed by Hawking (1955). The outstanding results led to its application against filariasis in dogs. It proved effective against *Dirofilaria immitis* (Hewitt, Kushner, Stewart, White, Wallace and Subbarow, 1947; Foley, 1950; Vaughan, 1952), but not so useful in combating *D. repens* (McGaughey, 1952). The drug has also proved effective against Epizootic Cerebrospinal Nematodiasis in sheep and goats (Shoho, 1952, 1954), and against ascarids in man (Loughlin, Rappaport, Joseph and Mullin, 1951), in ruminants (Das and Singh, 1955) and in dogs and cats (Kanegis, 1948).

The possibility that diethylcarbamazine might be effective against *Dictyocaulus viviparus* in calves was first mooted in an editorial footnote to an article on canine filariasis (Purchase, 1950) but there was little ground for such a hope. The larvae of *D. viviparus* use the blood stream as a means of passage to the lungs, but unlike the parasites responsible for filariasis in man and dog, they are not blood parasites. The worms responsible for the symptoms of husk have left the blood stream and are in the air passages of the lungs. Nevertheless in the absence of any effective treatment for so important a disease, any possibility was worth at least a preliminary investigation and, after a verbal report that a cow had recovered in spectacular fashion following oral dosage at the rate of 1 mg. per lb. body weight, an opportunity was sought to try the drug in a natural outbreak of verminous bronchitis.

EXPERIMENTAL

Farm Experiment

An outbreak of husk on a considerable scale was encountered in September, 1953, on a predominantly arable farm where large numbers of calves are purchased annually for rearing. The disease was severe in two groups of calves bought between September and December, 1952 at about a week old. They had been outside for 3 to 4 months and had been coughing for at least six weeks. When

first seen on September 8th, four deaths had already occurred and four calves were sinking fast. One calf was opened and found to be heavily infested with lungworms. The remaining 74 calves were divided into two random groups and one group was dosed orally at the rate of 1 mg. diethylcarbamazine per lb. body weight. Half the dosed group received a second dose at the same rate a fortnight later.

All the treated calves survived. Six of the controls died and five were killed on humanitarian and economic grounds. No benefit was attributed to the second dose. By that time all the treated and most of the untreated calves were improving. The improvement was sustained.

Controlled Infestation Experiment 1954

Following the apparent success of the farm experiment the drug was tested in calves artificially infested with lungworms in conditions of much stricter control and supervision. Dr. E. L. Taylor kindly arranged to rear 20 calves free from lungworm infestation at Weybridge, and Mr. J. F. Michel who was working on other aspects of the same disease administered the larvae before the calves left Weybridge. The calves were 7 to 12 weeks of age at the commencement of the experiment. Ten calves received 8,000 larvae, a dose considered likely to be fatal, and the other ten received 4,000 larvae. During the experiment they were housed in pens constructed in a disused aeroplane hangar and were fed on 3 lb. per head daily of a mixture of equal parts of flaked maize, crushed oats and linseed cake. Hay was always available.

Clinical Observations and Treatment

The calves were kept under close clinical observation for 94 days from the time of dosing with larvae. Slight coughing was first noticed on the 12th day, and was severe when group 4 were dosed by stomach tube on the 22nd day. A summary of the dosing and observations on the respiratory rates is given in Table I. The rate of

TABLE I

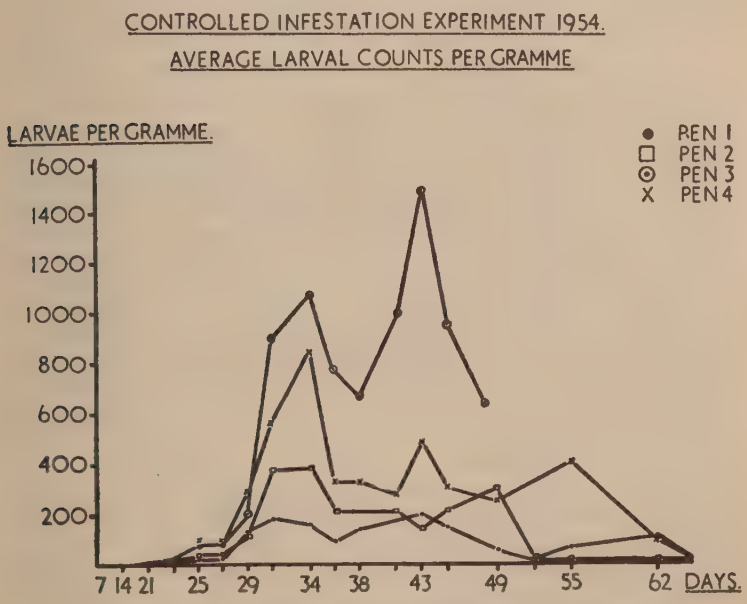
CONTROLLED INFESTATION EXPERIMENT 1954
RESPIRATION RATES AND DIETHYLCARBAMAZINE TREATMENT

Larval dosage	*Groups receiving 4,000 larvae*		*Groups receiving 8,000 larvae*	
Group	*1*	*2*	*3*	*4*
Treatment	*Control*	*200mg. 27th day*	*Control*	*200mg. 22nd day*
Av. Resp. Rate per min. (*a*) at treatment	75	75	90	90
(*b*) from treatment to 47th day	75–100	75–100	80–130	72–96

breathing was closely related to the severity of the disease until a late stage in fatal cases when breathing either became very erratic or became slower, accompanied by an expiratory grunt. Temperature recordings presented no useful information.

No benefit was seen in the treatment of Group 2 (Table I) as compared with Group 1. This may have been due to the light dosing with worm larvae or the late dosing with diethylcarbamazine. In Group 4 the drug relieved the respiratory stress and the larval counts from faeces were much less than in Group 3 (Fig. 1). Table 2 gives the record of calves which succumbed. Group 4 all outlasted Group 3 and three of them survived the experiment, though they received a major setback.

Fig. 1



The word "pen" is equivalent to the word "group" in the text.

Controlled Infestation Experiment 1955

The results of the 1954 experiment gave an indication that if used correctly, diethylcarbamazine might be effective in combating lungworm infestation. It was possible that the dose used on the farm was too low, or that repeated doses at much shorter intervals would be more effective. It was decided, therefore, to increase the dose to 25 mg. per lb. body weight and to repeat it on five successive days. Another possibility was that, as in filariasis, the drug was ineffective against adult worms and needed to be given early in the disease.

TABLE 2

DEATHS IN 1954 CONTROLLED INFESTATION EXPERIMENTS

Group	*No. of calf*	*Date of birth*	*Death*		*Weight on day of infestation*	*Weight of intact carcase*	*No. of lungworms*
			Date	*Day of exp.*			
2 4,000 larvae Treated	3341	1.7.54	6.10.54	50	116 lb.	90 lb.	678
3 8,000 larvae Controls	3324	26.6.54	18.9.54	32	153 lb.	—	1,190
	3334	1.7.54	1.10.54	45	110 lb.	92 lb.	240
	3343	1.7.54	3.10.54	47	130 lb.	—	1,216
	3323	26.6.54	4.10.54	48	145 lb.	100 lb.	954
	3317	23.6.54	4.10.54	48	107 lb.	98 lb.	891
4 8,000 larvae Treated	3325	26.6.54	22.9.54	36	124 lb.	95 lb.	1,116
	3318	23.6.54	22.10.54	66	96 lb.	—	139

One group (A) therefore, was to be dosed when the disease was sufficiently established to be recognised by a good stockman, and another (B) when it had reached an advanced stage, a third group (C) being left as controls. Each group consisted of five calves which were so allotted that the aggregate weight per group was approximately uniform. As the calves varied in size and much bullying occurred during the first few days, they were penned roughly according to size and not according to experimental group. Since they were all over three months old, the dose of larvae was increased to 10,000; this was given on the 4th October which is zero for the purpose of subsequent time reference. During this experiment the calves were weighed weekly and, as in 1954, were kept under close clinical observation throughout.

Feeding was as in the previous year except that the concentrates were increased from 3 lb. to 3½ lb. per day after the first month.

Clinical Observations

The disease ran a more rapid course than in the previous year. Slight coughing was first noticed on the sixth day, that is in half the time, but characteristic "husking" with the head extended for a strong expiratory effort was not seen until the 13th day. Administration of the drug to Group A took place on the 14th to 18th days inclusive. The dose was calculated for the known weight of each calf and administered by stomach tube. Groups B and C suddenly became very ill on the 19th day, and a five-day course of treatment of Group B was commenced. Detailed clinical observations were commenced on the 14th day and continued until the 50th day, by which time it was quite clear that Group A were thriving and that no calf from Group B or C was ever likely to thrive. Weekly weighing continued until the 113th day when the experiment was terminated.

The difference between Group A and the other two groups was pronounced and sustained. By the third day of dosing Group A was clearly showing improvement. The animals were brighter, more alert and their breathing was less obvious. Reference to Table 3 shows that respiratory rates were highest in Group A before treatment and fell as soon as treatment started, whereas in Group B there was no such effect. In the Table the respiration rates of Group A are averaged for convenience of day to day comparisons. Because of laboured breathing, averages in Groups B and C would be misleading. Body temperatures in Group A were above normal before treatment and fell to normal, or very near normal, during treatment. No similar effect occurred in Group B.

Weight gain (Table 4) was sustained, if not very steady, in Group A, whereas in both groups B and C the individual weights remained fairly static or decreased until death supervened.

Deaths (Table 5). Four of the control calves and two of Group B died between the 19th and 24th days. Apart from calf 3760 (Group

TABLE 3

RESPIRATORY RATES IN 1955 CONTROLLED INFESTATION EXPERIMENTS FROM 14TH TO 26TH DAY

Day		*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*
Group A		*	*	*	*	*								
Treated early	3740	122	82	88	66	44	72	100	76	60	32	60	38	50
	3741	126	75	82	68	60	84	96	100	88	66	66	56	65
	3714	88	74	104	68	60	80	96	126	88	50	60	60	64
	3799	140	60	80	42	62	50	80	72	68	48	34	22	30
	3797	104	84	66	72	32	84	60	80	80	40	60	42	57
	Average	116·0	75·0	84·0	63·2	51·6	74·0	86·4	90·8	76·8	47·2	56·0	43·6	53·2
Group B							*	*	*	*	*			
	3745	92	76	64	80	64	112	92	104	116	76	96	72L	96L
	3742	84	68	80	62	72	112	96	120	124	104	108	110L	88L
Treated late	3782	60	64	68	78	96	96	80L	Died 21st day					
	3791	80	56	78L	78	56L	64L	70L	68L	60L	40L	Died 24th day		
	3796	80	68	76	76	96	110	100	100	60L	46L	42	46	50
Group C														
	3760	92	84	76	62	66	92	108	100	84	80	64	28L	80
	3762	100	108	104	112	114	132	140	128L	86L	60L	Died 24th day		
Control	3787	80	80	76L	68	108	60L	Died 20th day						
	3792	88	80	88	92	64L	Died 19th day							
	3785	120	104	116	100	128	88	96	100L	Died 21st day				

* = Group dosed with diethylcarbamazine
L = Laboured breathing.

C) no animal in Group B or C showed signs of recovery. After treatment of Group B, calf 3760 stood out as the best of the two groups and often looked as good as those in Group A. Its respiratory rate and sounds were much healthier than those of the other calves in Groups B and C and it easily outlasted them.

Larval counts on faeces samples were carried out at frequent intervals. Production of larvae was almost completely suppressed in Group A. Four of the controls were dead by the 24th day, too early for any appreciable production of larvae. The remaining calf in this group was quite exceptional in many respects and so its larval count is not representative. The larval counts in Group B were also interfered with by early death, but the two animals that lived long enough to pass the peak period had very low larval counts compared to the previous year's results, the peak figure being only 80 larvae per gramme on the 40th day. The outstanding feature of the experiment was not so much that the calves in Group A survived when all others died, but that they put on weight and thrived.

Challenge with Further Infestation

The main feature of this work was to test the efficacy of diethylcarbamazine in treating husk, but the idea of challenging the immunity of recovered animals was also studied. Since recovery after treatment was so complete it was decided to dose some of the calves with a further 10,000 lungworm larvae on the 66th day. From Group A, the second and third calves by weight (3740 and 3741) were chosen for dosing. Calf 3760 was also dosed with larvae for comparison since this calf had already suffered an attack of the disease and had not received any treatment. The true "control" for the experiment would have been some calves reared free from lungworms, but no such animals were available.

Control calf 3760 died on the 78th day of the experiment, only thirteen days after the challenging dose of worm larvae. The appearance of the lungs was typical of the long term effects of heavy lungworm infestation. No worms were found in the lungs and the death cannot be considered to be in any way associated with the challenging dose of larvae. The two calves from Group A which were challenged showed no ill-effects. Faecal larval counts were carried out on nine occasions between the 13th and 47th days after the challenging dose (78th and 113th days of the whole experiment). On the 21st and 26th days, 0·1 larvae per g. were found in the faeces of calf 3741. These were the only positive counts and on the second occasion the larvae appeared to be dead.

Pathological Changes in Controlled Infestation Experiments

Post-mortem examinations were carried out on all the fatal cases and it is hoped to publish details in a later article. The salient features only are given here. Consolidation of the apical and

TABLE 4

WEEKLY WEIGHTS OF CALVES IN CONTROLLED INFESTATION EXPERIMENTS 1955

Day		*0*	*8*	*15*	*22*	*29*	*36*	*43*	*50*	*57*	*64*	*71*	*79**	*85†*	*92*	*99*	*107**	*113†*
Group A																		
Treated early	3740	171	167	176	199	209	222	226	236	—	262	270	267	287	280	316	320	352
	3741	159	163	161	165	185	194	220	203	224	230	241	224	224	257	258	226	292
	3714	188	191	191	210	224	224	254	252	252	274	253	291	289	316	308	317	322
	3799	122	120	108	121	144	149	171	163	178	188	185	206	194	219	215	222	245
	3797	131	135	134	143	157	170	202	189	204	221	213	234	235	256	258	271	289
Average		154·2	155·2	154·0	167·6	183·8	191·8	214·6	208·6	—	235·0	232·4	244·4	245·8	265·6	271·0	291·2	300·0
Group B																		
Treated late	3745	158	161	166	155	168	157	189	160	Died 57th day								
	3742	179	185	185	157	157	149	161	150	Died 53rd day								
	3782	136	142	128	Died 21st day													
	3791	113	116	119	88	Died 24th day												
	3796	160	179	147	134	141	Died 33rd day											
Average		149·2	156·6	149·0														
Group C																		
Control	3760	185	184	185	162	169	184	189	194	201	210	203	Died 78th day					
	3762	176	183	184	157	Died 24th day												
	3787	153	161	161	Died 20th day													
	3792	106	105	97	Died 19th day													
	3785	139	143	143	Died 21st day													
Average		151·8	155·2	154·0														

* = 8 days from last weighing
† = 6 days from last weighing.

cardiac lobes was the only consistent finding. In the diaphragmatic lobe consolidation varied considerably in extent, but was always present near the ventral margin, while emphysema was more frequent in the dorsal part.

The five calves of Group A (1955) had survived the attack following treatment and their lungs were examined after slaughter on the 113th day. The lungs of two calves appeared normal. In the other cases there was some consolidation of the apical and cardiac lobes, but only in one was there any consolidation in the diaphragmatic lobe. Very little emphysema was found. All five pairs of lungs were ribboned and examined for adult worms with negative results, even though two had been challenged with a further dose of larvae on the 60th day. Saline washings of the opened lungs revealed no larvae.

DISCUSSION

Lungworm infestation results in the early stages in bronchitis, but later consolidation develops, probably associated with secondary bacterial invasion (though this is not easy to demonstrate post-mortem) and the temperature rises. Thereafter, if the calves have been brought in and are being well cared for, a critical period arises in the group with distinct similarity to the crisis of pneumonia in its classic form in man. A varying proportion of the calves die, but quite suddenly deaths cease and all or nearly all the survivors commence to improve. This sudden change easily leads to false conclusions regarding the usefulness of drugs in treating this disease. Treatment is usually supplemented by improvement in the husbandry but there is often a tendency to attribute the animals' improvement to the drug rather than the husbandry. It is, therefore, particularly true of this disease that only a controlled experiment affords a reliable test of any therapeutic agent.

Before the experiments here described were commenced, it was hoped that diethylcarbamazine might be of help while the disease was purely a parasitic one, but it was feared that once pneumonia had set in with probable bacteriological complications the drug would be of less value. It was therefore surprising to find in the experiments on the farm that the value of the drug appeared to lie in the prevention of death. The results of the controlled infestation experiments, however, lead rather to the conclusion that the drug is most effective when the worms are young. The small dose used in the 1954 experiments on the 22nd day might have been more effective if given earlier, for although dosing commencing on the 19th day in 1955, failed to prevent the death of any of the five calves in Group B it did seem to prolong the lives of some of them. Indeed, a comparison of the controlled infestation experiments in both years suggests that the effectiveness may be associated more with time of dosing in relation to the date of infestation than to the size or frequency of dosing. Against this view is the fact that the oldest calves

TABLE 5

DEATHS IN 1955 CONTROLLED INFESTATION EXPERIMENTS

Group	*No. of calf*	*Date of birth*	*Death*		*Weight on day of infestation*	*Weight of intact carcase*	*No. of lung worms*
			Date	*Day of exp.*			
B Treated late	3782	14.6.55	25.10.55	21	136 lb.	103 lb.	955
	3791	23.6.55	28.10.55	24	113 lb.	80 lb.	431
	3796	30.6.55	6.11.55	33	160 lb.	125 lb.	75
	3742	25.5.55	26.11.55	53	179 lb.	135 lb.	45
	3745	25.5.55	30.11.55	57	158 lb.	135 lb.	5
C Controls	3792	23.6.55	23.10.55	19	106 lb.	79 lb.	421
	3787	22.6.55	24.10.55	20	153 lb.	138 lb.	138
	3785	20.6.55	25.10.55	21	139 lb.	115 lb.	378
	3762	7.6.55	28.10.55	24	176 lb.	135 lb.	2,157
	3760	4.6.55	21.12.55	78	185 lb.	180 lb.	—

in the farm experiment had been coughing to an extent sufficient to make the farmer bring them in at nights, fully six weeks before an apparently effective though small dose of diethylcarbamazine was given. The explanation of this difference may lie in the fact that the artificially infested calves received the worm larvae in one dose, whereas the calves on the farm would be picking them up daily, possibly in increasing numbers, even after being brought in at night. If immature worms were present in the lungs in large numbers and the drug destroyed most of these, the pathological process might change immediately from one of destruction of the lung tissue to one of resolution. If this hypothesis were to prove correct the value of the drug in field outbreaks of husk would be greatly increased.

The question of whether this drug would be effective against a less heavy infestation remains unanswered by these experiments. Dosing the less heavily infested calves on the 27th day proved quite useless in the 1954 experiment, but treatment one or two weeks earlier might have had very different results. The greater effectiveness of early dosing is in line with the ease with which the larvae are removed from the blood in filarial infections of the cotton rat (Hawking, Sewell and Thurston, 1948, 1950) and the dog (Hewitt *et al*, 1947), and their destruction by phagocytosis, while the adult worms are much more resistant. The passage of *Dictyocaulus* larvae from the mesenteric lymph glands to the lungs is stated by Cameron (1951) to take place between the 4th and 10th day after ingestion. Though this is before the earliest dosing tried in these experiments the worms are still not adult when arriving at the lungs and their presence would in itself attract phagocytes to the centre of irritation. The opsonin-like effect of diethylcarbamazine in the destruction of Litomosoides in the cotton rat described by Hawking *et al.* (1948), may therefore also apply in the destruction of *D. viviparus*.

Though the drug, particularly in 1955, depressed the reproductive rate of the parasites, the peak larval counts came at about the same time (between the 45th and 50th days) as in infestation experiments where no attempt was made at treatment (Michel, 1955; Jarrett, MacIntyre and Urquhart, 1954). No toxic effects were observed during treatment, either from the drug itself or from the killed worms.

CONCLUSIONS

In a natural outbreak of husk, oral dosage of diethylcarbamazine at the rate of 1 mg./lb. prevented deaths from parasitic bronchitis in 37 calves 9 to 12 months old; 11 of 37 control calves died or had to be destroyed.

In experiments with controlled worm infestation in calves under five months of age, large doses of 25 mg./lb. daily for five days, given 14 to 18 days after heavy infestation, prevented death and the calves made reasonable weight gains. Treatment at later stages was less effective and tended to delay death rather than to prevent it.

Diethylcarbamazine depressed larval counts in the faeces of calves affected with parasitic bronchitis.

Further experiments are needed to discover the optimum dose, the effectiveness of the drug in moderate lungworm infestations and the period of the disease during which the drug is effective.

ACKNOWLEDGMENTS

I am indebted to Messrs. Burroughs Wellcome for the supplies of "Banocide" (Diethylcarbamazine) for this work, and to Dr. R. F. Montgomerie and Mr. S. L. Hignett of the Wellcome Veterinary Research Station, Frant for their helpful interest. Thanks are equally due to Dr. E. L. Taylor and Mr. J. F. Michel without whose help the work could not have been undertaken, and to the staff of the Veterinary Investigation Centre at Wolverhampton who have been responsible for much of the tedious detail involved.

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EMBOLIC DISPLACEMENT OF VIABLE TESTICULAR TISSUE IN A BROWN LEGHORN COCKEREL

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INTRODUCTION

Spontaneous displacement of viable non-neoplastic cells from one organ to another is a phenomenon of sufficient rarity to warrant publication of the details, even though the aetiology is not fully understood.

CASE RECORD

A cockerel (D. 1514) from the flock of the Poultry Research Centre was killed when ten months old as it had been losing condition for some time. At post mortem examination a large irregular thrombus was found firmly adhering to the endocardial lining of the left ventricle. Almost half the left testis was necrotic; there was a distinct line of demarcation between healthy and dead tissue. The spleen and liver were slightly enlarged and the latter was congested. A few petechial shock haemorrhages were present in the coronary groove. No other abnormality was seen on macroscopical examination.

Histological Examination

Histologically, the endocardial vegetation was found to be made up mainly of a mass of caseous necrotic tissue; it contained large bacterial colonies, particularly at the periphery. This necrotic mass was bordered by an acute inflammatory reaction which was characterised by severe haemorrhage and the involvement of numerous heterophils. This area was enclosed in turn by a layer of multinucleated giant cells and a narrow zone of rather dense granulation tissue rich in hyalin. The left testis showed the typical picture of an anaemic infarct. The lumen of one of the larger branches of the testicular artery was completely occluded by a bacterial thrombus, which was firmly adherent to the severely inflamed and partly necrotic arterial wall. The seminiferous tubules normally supplied by this vessel showed advanced coagulative necrosis, but were clearly demarcated from the normal portion of the testis. Pyknosis, affinity for eosin and loss of structure were the characteristic features of the necrotic germinal epithelium. Few seminiferous cells remained intact in the affected tubules; in many tubules the epithelium was converted into an almost homogeneous eosinophilic mass. The mature spermatozoa within the lumina of the tubules were apparently unaffected by the necrotic process. The rather inactive spleen showed severe amyloidosis and haemosiderosis. Miliary foci of fibrinoid necrosis were scattered throughout

the substance of the somewhat fatty liver, which also showed hypertrophy of the sinusoidal endothelial cells. The most striking abnormality, however, was to be seen in one of the larger branches of the portal vein (Fig. 1), the lumen of which was almost completely filled by a very loose riding thrombus, one limb of which extended into a small branch vessel. Low power magnification was sufficient to show, within the substance of the thrombus, large round cells, obviously not blood cells, which were readily recognized with high power magnification (Fig. 2) as seminiferous cells. All stages of spermatogenesis were represented. Only a very small number of spermatocytes showed pyknosis and all the other cells, particularly the spermatids and sperms appeared healthy and viable. A number of the smaller veins in the liver, the kidney (and perhaps a number of other organs unfortunately not retained for histological examination) contained similar seminiferous cells in smaller numbers and apparently not within a thrombus but free in the circulation. An accumulation of similar cells, including fully developed sperms was found in some spaces of the otherwise normal myocardium (Fig. 3). Since they also contained erythrocytes, these must be considered as capillary spaces although no endothelial lining could be demonstrated.

DISCUSSION

It is not easy to offer a fully satisfactory explanation for the mechanism involved in the dissemination of these viable germinal cells to non-testicular organs. If one accepts their testicular origin, the method of their dislodgement has to be explained. There can be no doubt that such a large cell mass as that found within the branch of the portal vein could not have been carried directly from the testes, since no anastomosis of portal and testicular veins is known to exist. Paradoxical embolism can also be ruled out on anatomical grounds. This suggests, therefore, that these testicular cells must have been dislodged into the blood stream singly or in very small groups.

How these cells became first detached is itself difficult to explain. Although one testis was extremely necrotic, following severe infarction, embolism is not a generally recognized sequel of necrosis, albeit the vascular endothelium itself is affected. Trauma is known to give rise occasionally to embolism in cases of liver rupture, bone fracture etc. In the case under discussion neither testis showed any signs of traumatic injury. Active invasion of blood vessels, such as one is accustomed to see in anaplastic and rapidly growing neoplasms or the frequent involvement of spermatic vein, vena cava or even the right chambers of the heart in testicular teratomata (Willis, 1952) cannot be held responsible for embolism in this case, since neither of the testes was tumorous. Metastatic chorioepitheliomata arising from apparently healthy uteri have often been reported and the literature is covered by Willis (1953). The

liberation into the blood stream of viable cells from a normal testis might at first sight appear analogous. On the other hand Novak and Koff (1930) have demonstrated by curettage in some clinical cases of metastatic chorioepithelioma the pre-existence of a primary uterine tumour, which later disappeared, leaving no trace at autopsy. Non-neoplastic chorionic villi can often be found as emboli during normal pregnancy and it must be assumed that they are of post-traumatic origin, but are, as Nicholson (1950) points out "always in a more or less degenerate condition" and could not, therefore, give rise to active metastases.

A search of the literature has indicated that apparently authentic cases of viable metastases from normal organs have only been reported for the thyroid (Eberth, 1872; Riedel, 1893; Odersfeld and Steinhaus, 1901). Becker (1900) described a tumour, composed of histologically normal thyroid tissue, in the supraclavicular fossa of a patient with a normal thyroid gland. This case must however be interpreted as hyperplasia of an accessory thyroid, and not metastasis of normal tissue. Nicholson (1950) in analysing the extremely interesting case of v. Eiselsberg (1894) of a physiologically functional metastasis of a thyroid tumour, points out that, whereas normal thyroid emboli may lodge in bone, the embolus will only grow if the thyroid is deficient. Experimental evidence supports this (Maximow and Bloom, 1942; Cowdry, 1932). Again, Manasse (1894) discovered emboli of adrenal tissue in the veins of the normal gland; the possibility of adrenal metastases may, therefore, be accepted, but proliferation may be expected only in adrenal deficiency. The close association of the parenchyma and the venous circulation in both thyroid and adrenal glands is said to facilitate the formation of emboli in these organs (Pick, 1901).

The foregoing observations raise the question of the survival in the circulation of seminiferous cells. Since all but a few of these cells show no degenerative changes they must have been liberated from still healthy seminiferous tubules. It is impossible to say whether they had reached the circulation just before death and would eventually have died if the bird had not been killed, or whether they had actually survived within the blood stream for some time and perhaps multiplied and developed. This latter view is the more likely since it would explain the appearance of fully viable, multiplying and developing cells within the large liver thrombus, a thrombus which, as pointed out above, was too large to have been carried in toto from the testes to the liver. Testicular, like adrenal and thyroid tissue, is readily transplantable and it is not impossible that the milieu of the portal vein was quite favourable for the development of these cells.

Although one may accept the theory of Swift (1913, 1915, 1916) and Dantschakoff, Dantschakoff and Bereskina (1931) of the blood-migration of primordial germ cells, Willier, Gallacher and Koch (1937) have shown that these cells require for their further develop-

ment a specific sex cord environment. Development and full maturation of primordial germ cells within the circulation is, therefore, more than unlikely, particularly since all investigators agree that these cells have disappeared from the circulation by the time the embryo has reached the 25 somite stage. An explanation of this phenomenon on the basis of displaced primordial germ cells is, therefore, not acceptable.

CONCLUSIONS

A description is given of a generalised embolic dissemination within the venous blood stream to a number of non-testicular organs of apparently viable seminiferous cells, including fully developed spermatozoa. The possible aetiology of this phenomenon is discussed.

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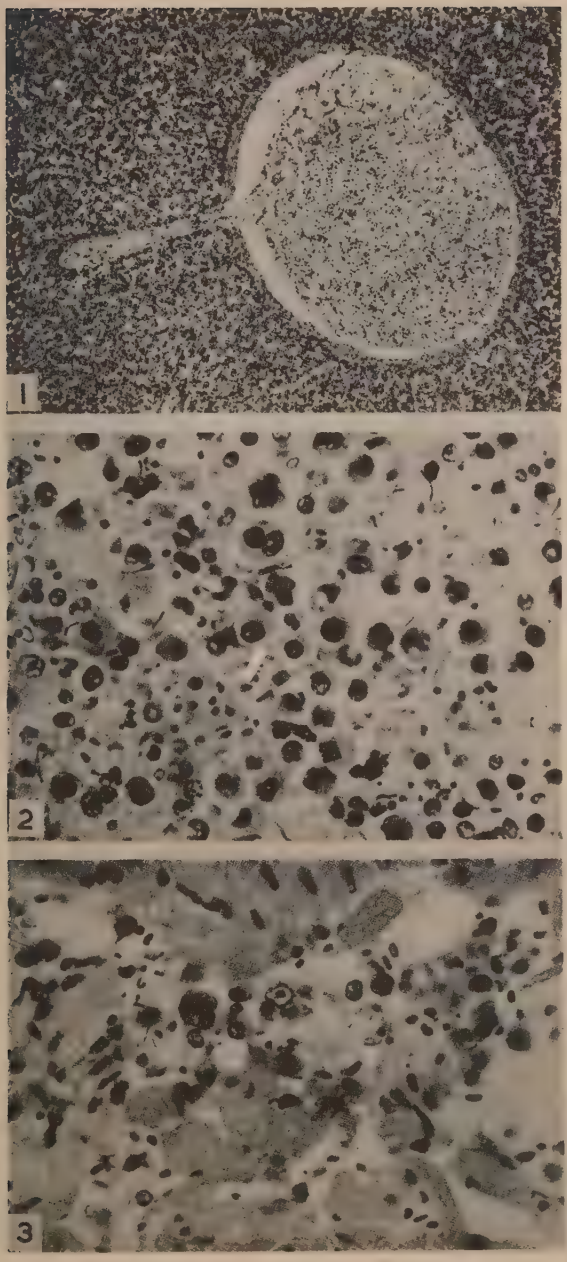
LEGENDS TO ILLUSTRATIONS

Fig. 1. Large riding thrombus in the portal vein. H. & E. × 30 approx.

Fig. 2. Portal vein thrombus showing germinal cells in all stages of development including mature spermatozoa. H. & E. × 350 approx.

Fig. 3. Numerous germinal cells including mature sperm may be seen within the capillary space of the myocardium. H. & E. × 350 approx.



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MUCOSAL DISEASE COMPLEX

By

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INTRODUCTION

During the past few years a disease affecting the alimentary tract of calves has been investigated. While the naturally occurring infection closely resembled the mucosal diseases described by Ramsey and Chivers (1953) and other workers in North America, the condition produced experimentally was similar to virus diarrhoea (New York) described by Baker, York, Gillespie and Mitchell (1954).

NATURAL DISEASE

Symptoms and Epidemiology

Five outbreaks of the natural disease which occurred between February and June in different years have been investigated. The disease occurred in groups of calves from four to eighteen months of age and, although younger and older cattle had either direct or indirect contact with the infected animals, no apparent spread occurred.

Clinically the condition was characterized by lack of appetite, frequent drinking, mucoid nasal discharge and diarrhoea. Pyrexia occurred but was not a constant feature during illness. The muzzle was encrusted with dried discharge and accumulated debris, and coughing and laboured breathing were sometimes observed. The eyes were not involved. Diarrhoea was frequently followed by tenesmus and the voiding of blood-stained mucus.

In some cases the mouth showed changes varying from general hyperaemia of the epithelial lining, peridental cyanosis around the base of the incisor teeth, to shallow ulceration on the lips, buccal epithelium, tongue and hard palate. Caseous debris was frequently adherent to the dorsum of the tongue and ulceration sometimes extended into the nares. In one outbreak ulceration was observed between the claws of affected animals. Body condition was rapidly lost and death occurred three to twenty-one days after the first appearance of symptoms. There was up to 60 per cent morbidity and the mortality was high, as will be observed from Table 1.

Post-mortem Lesions

The lesions varied from a mild catarrhal enteritis to severe haemorrhagic gastroenteritis with ulceration which sometimes extended from the nares and oral cavity to the rectum. In some instances the lesions in the nares and oral cavity extended into the pharynx and larynx, and discrete shallow ulcers or dark-coloured longitudinal lesions composed of cellular debris with lymphocytic

infiltration were found in the oesophagus. The epithelium of the rumen, reticulum and omasum was yellowish and covered with an easily removable, necrotic layer. The contents of the omasum were often wet. Discrete haemorrhages and ulcers were present in some cases in the abomasum. The extent of the haemorrhages and ulceration varied and on occasions were found throughout the small and large intestine, caecum and rectum. The haemorrhages in the large intestine and rectum gave the effect of 'striping'. The lumen of the small intestine commonly contained a glairy, tenacious mucus in which clots of blood were sometimes present. Peyer's patches were dark and congested, the lymph nodes were frequently enlarged and on section were wet and tumified; many were cherry-red in colour. The haemo-lymph nodes were also prominent. Except for occasional ulceration between the claws, lesions were not found on any other part of the body.

TABLE I

Outbreak	*Number of animals in the affected group*	*Mortality*
1	13	8
2	8	3
3	4	2
4	12	2
5	50	21
Total	87	36

Cultures of tissues for bacteria, using aerobic, anaerobic and ten per cent CO_2 atmospheres, have shown no significant growth.

EXPERIMENTAL DISEASE

The condition was transmitted by inoculation of citrated whole blood, plasma, lymph node and brain suspensions, and lymph-node suspension intravenously and intraglandularly with tissues obtained from a calf in the first outbreak. Suspensions were prepared by grinding tissues with sterile sand, filtering through gauze and centrifuging at 10,000 r.p.m. for one hour. The supernatant fluids were used for inoculation.

Filtrates of a lymph-node suspension prepared by freezing and thawing five times and passing through a Gradocol membrane of 680 mμ A.P.D., were infective. A 590 mμ A.P.D. filtrate of a lymph-node suspension and plasma was also infective.

Reference to Table 2 shows that there was little difference in the susceptibility of laboratory-bred and bought-in calves. Of 112 calves inoculated, ninety-five, or 84·8 per cent, reacted to the first inoculation, and seventeen, or 15·2 per cent, failed to react. Thirty-one calves positive to the first inoculation were re-inoculated; twenty-seven showed no reaction and four gave a mild, positive reaction. Fourteen animals which did not react to the first inoculation were re-inoculated; six reacted, and eight failed to react at all. (Table 3). In no case did death occur in experimental calves due to the inoculation of infective material.

TABLE 2

INOCULATIONS OF BOUGHT-IN AND LABORATORY-BRED ANIMALS

Origin of calves	*Number inoculated*	*First inoculation reaction*	*Second inoculation reaction*	
Home bred	40	+ve	N.I.	
	9	+ve	—ve	
	3	—ve	—ve	
Total	52			
Bought in from outside farms	24	+ve	N.I.	
	3	—ve	N.I.	
	18	+ve	—ve	Two animals —ve to the first inoculation were not challenged as both were pregnant when inoculated. One aborted and both were killed. The third animal was used to test another strain of virus.
	4	+ve	+ve	
	6	—ve	+ve	
	5	—ve	—ve	
Total	60			

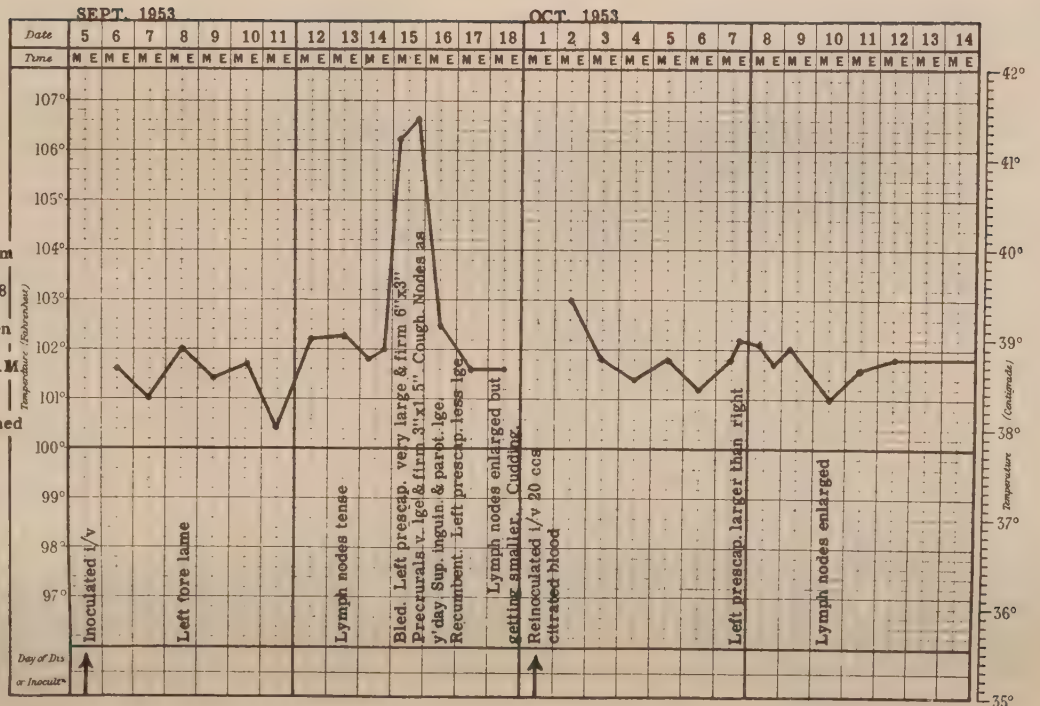
N.I.=Not Inoculated

Clinical Symptoms

The experimental disease differed from the natural cases investigated in that it was very mild. Characteristically there was pyrexia of short duration (one to three days) with a mucoid nasal discharge from six to ten days after inoculation. Other signs were inconstant but anorexia and coughing were noted. Diarrhoea occurred a few days after the temperature reaction. Enlarged lymph nodes were observed. In a few animals ulceration of the oral cavity commenced a few days after the pyrexia and persisted for seven to ten days.

Post-mortem Findings

Eight animals were killed, four at the height of the temperature reaction, one at three, one at eight, and one at twelve days after the



Calf 2813
Friesian Steer
Box 10
About 4 months old

Remarks
Inoculated i/v 6 ccs. 680 mμ Gradocol membrane filtrate from lymph node saline suspension of calf 2348
Cultures - no growth.
L. n. suspension frozen & thawed 5 times, centrifuged 10000 R.P.M for 1 hour and supernatant filtered.
Culture of blood obtained on 15/9 - no growth

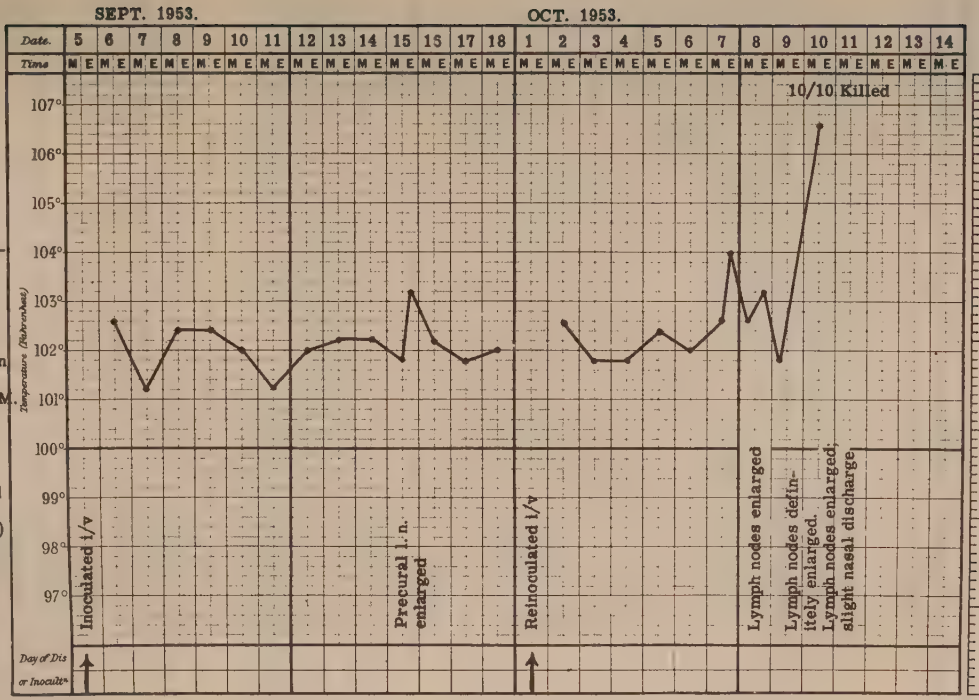
1/10/53 Reinoculated i/v 20 ccs citrated blood 28/3 (15/9) 2811 (13/9) and 2337 (2/9)

Calf 2814
Friesian Steer
Box 10
About 4 months

Remarks

Inoculated i/v 5·ccs. 680 mμGradocol membrane filtrate from saline suspension of lymph nodes of calf 2348.
Cultures - no growth.
L. n. suspension frozen and thawed 5 times, centrifuged 10000 R.P.M. for 1 hour and supernatant filtered.

1/10/53. Reinoculated i/v 20 ccs. citrated blood from 2813 (15/9) 2811 (13/9) and 2337 (2/9)



temperature reaction. Very few changes were found. Two showed ulcers in the alimentary tract, with tenacious mucus and clotted blood in the lumen of the small intestine. Peyer's patches were markedly congested in a few cases. A calf killed eighty-eight days after reacting showed no abnormalities.

TABLE 3

SUMMARY OF INOCULATIONS OF CALVES WITH THE LABORATORY STRAIN OF VIRUS

First inoculation	*Reaction*	*Re-inoculated*	*Reaction*	*Number of calves*	*Interval between first and second inoculations*				*Not reinoculated*
					2–4 weeks	*1–2 months*	*2–4 months*	*Over four months*	
95	+	31	+	4	2	2			64
			—	27	8	3	13	1 at 8 months 1 at 13 ,, 1 at 22 ,,	
17	—	14	+	6	2	3		1 at 7 months	3
			—	8	5		1	2 at 7 months	
112		45		45	17	8	14	6	67

Duration of Immunity

Reference to Table 3 shows that of thirty-one animals which had reacted, eight were immune two to four weeks, three 1 to 2 months, and thirteen 2 to 4 months later. A further three were immune when tested, one at eight, one at thirteen and one at twenty-two months after the first inoculation.

Age Susceptibility of Bovines

One calf inoculated when one week old showed no reaction. It subsequently reacted when reinoculated six months later. About half of the adult animals inoculated have shown a mild temperature rise to 103·6°F. about seven days after inoculation, followed a few days later by diarrhoea lasting one to three days.

Two heifers when six-and-a-half months pregnant showed no reaction following inoculation of infective material. Sixty days later one animal aborted a dead foetus. Suspension of aborted foetal tissues failed to provoke a reaction when inoculated into calves. The second heifer was slaughtered and found to have a normal foetus. Tests for *Brucella abortus* and *Vibrio fetus* were negative.

Transmission by Contact

Three healthy animals placed in contact with natural cases of the disease contracted infection and reacted within the incubation

time of the experimental disease. Attempts to transmit the experimentally induced condition failed in one of two trials. One animal reacted eight days after being in contact and also when inoculated two months later. A second contact animal showed no reaction and did not subsequently react to inoculation.

Properties of the Infecting Agent

Citrated whole blood stored for six months at 4°C. remained infective. Lymph nodes stored at —20°C. for one month, then ground and suspended in saline were infective. A suspension of brain and lymph nodes obtained from a calf eleven days after the temperature reaction was infective. Tissues obtained from a calf killed eighty-eight days after reaction were not infective.

Sheep, Goats and Pigs

The agent has been passaged serially three times in sheep and goats respectively. Blood from the third passage of these animals was infective for calves. In sheep and goats temperature rises were recorded from five to eight days after inoculation. Lack of appetite and diarrhoea have been noted in sheep.

Two pigs immune to swine fever remained healthy, but a slight rise of temperature was recorded on the fifth and sixth days after inoculation.

Transmission to Laboratory Animals, including the Embryonated Egg

Attempts to adapt the agent to rabbits, guinea-pigs, mice, suckling mice, hamsters, ferrets and pigeons have failed. No reaction was observed after inoculation.

Six and ten-day-embryonated hen eggs inoculated by the yolk-sac and chorioallantoic routes did not show any abnormal reaction. Two attempts to infect calves by inoculation of egg tissues failed.

DISCUSSION

The natural disease has features in common with several other conditions, notably malignant catarrh, coccidiosis, corrosive poisoning and cattle plague. These must be considered in differential diagnosis.

Eye involvement usually occurs in malignant catarrh and generally the disease tends to be sporadic. The agent of malignant catarrh differs from that present in the outbreaks recorded here in that it will not withstand freezing and thawing (Anon, 1941). It has also been shown (Piercy, 1952, 1953) that malignant catarrh is not transmissible by filtrates and that the agent is fragile and will not survive storage at various temperatures favourable to the survival of the agent in our cases. It was shown by Daubney and Hudson (1936) that the agent of malignant catarrh of cattle could be established in rabbits.

In respect of common poisons availability and chemical tests would establish a diagnosis.

In coccidiosis involvement of the anterior portion of the alimentary tract would not be expected. Diagnosis of this condition would be supported by a response to treatment.

Differentiation between mucosal disease and rinderpest would depend in the first instance on cross-protection tests.

Hudson (1950) showed that a diagnosis of Tick-borne Fever could be made by finding cytoplasmic inclusion bodies in the white blood-corpuscles during the febrile phase. No inclusion bodies have been demonstrated in mucosal disease and cross-protection tests have shown that the causal agents are immunologically distinct.

During recent years there have been several accounts of mucosal diseases affecting the alimentary tracts of cattle in North America, by Ramsey and Chivers (1953); Schipper, Everleth, Shumard and Rickards (1955); Nielson, Horney, Hulland and Roe (1955); Pritchard, Taylor, Moses and Doyle (1956). The clinical accounts and post-mortem findings closely resemble those seen in England.

Dow, Jarrett and McIntyre (1956) described three types of natural disease in Scotland; mild acute, severe acute and chronic. The condition described here resembles their severe acute disease. Material submitted by them from a case of disease in 1955, and tested by cross-protection tests, was shown to contain an agent immunologically identical to that isolated from the first outbreak investigated at this laboratory.

Virus diarrhoea (New York), first described by Olafson, MacCallum and Fox (1946), differs from the natural disease encountered here in that it principally affects adult animals and has a low mortality. However, the experimental disease described by Baker *et al.* (1954) is very similar to that encountered here. Further, the agents are similar in their failure to be propagated in small animals and embryonated eggs. Virus diarrhoea (New York) was at one stage suspected to be rinderpest, but Olafson and Rickard (1947) and Walker and Olafson (1947) showed by cross-protection tests that the two conditions were unrelated.

It is of interest to note that a disease of cattle described by Simonds and Brown (1871) in Kent was clinically suspected to be cattle plague. After investigation, they suggested that the condition was due to acorn poisoning. In 1934, Jones suggested that coccidiosis was a more appropriate diagnosis for the acute form of acorn poisoning. A gastro-enteritis syndrome described by Völker (1950) was attributed to acorn poisoning; he added that the most serious outbreak resembled cattle plague. In view of the similarities of the diseases described by these writers and the unlikely possibility that the entity described in this article is a new disease, it would appear desirable to attempt to establish the relationship between some of the ill-defined conditions of haemorrhagic gastro-enteritis of cattle and the mucosal disease complex.

The outbreaks investigated here all occurred in closed herds. It is possible that a mild condition resembling the experimental

disease is prevalent. If this were so, it could easily be overlooked under ordinary farm management conditions. It might well be that if infection were introduced into a closed herd the stock, being highly susceptible, would react severely.

CONCLUSIONS

A mucosal disease of bovines is described. It occurred in calves and a high mortality in affected animals was common. Clinically there was high temperature with mucoid nasal discharge, scour, and sometimes blood-stained faeces. On post-mortem examination the digestive tract usually showed lesions varying from a mild enteritis to a haemorrhagic enteritis with free blood in the lumen of the bowel. Haemorrhages occurred in the abomasum. Ulceration of the mouth was often present and might extend into the pharynx and oesophagus. Lymph nodes were sometimes enlarged and showed perinodal oedema. A patchy, cherry-red appearance of the lymph nodes was often found on section, and the haemolymph nodes were prominent.

The experimental condition was very mild and was characterized by an incubation period of six to ten days, when a high temperature occurred and lasted about twenty-four hours, often accompanied by a mucoid nasal discharge. A diphasic temperature rise has been observed in some cases. Lymph nodes were often enlarged. Scour and coughing were inconstant features of the experimental condition. Ulceration of the oral mucosa occurred infrequently.

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THE EFFECTS OF A DIET DEFICIENT IN VITAMIN A ON THE DEVELOPMENT OF THE SKULL, OPTIC NERVES AND BRAIN OF CATTLE

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INTRODUCTION

Moore, Huffman and Duncan (1935) described a form of blindness in the calf due to constriction of the optic nerve at the point where it passes through the optic foramen. The condition could be prevented by a diet containing carotene. Similar observations were later made by Mellanby (1938, 1941 and 1944), who found constriction and degeneration of the optic, auditory and trigeminal nerves in young dogs fed on diets deficient in vitamin A. The optic nerve was subjected to pressure and distortion in some parts of its course owing to abnormal and excessive bone growth. In deficient dogs the optic canal was longer than normal and the nerve, after emerging from the canal on to the base of the skull, passed through a sharp and deep triangular groove, instead of the normal oval passage. This caused pressure and subsequent degeneration. The anatomy of this area in the ox skull is rather similar to that of the dog, although the optic groove is normally flat. It is known that the optic canal is an area subjected to rapid changes during growth (Moore, 1941).

Wolbach (1947), who reviewed the effect of vitamin A deficiency, mainly from observations made on rats, produced further evidence of the interference with normal bone growth, and stressed the importance of deficiency in retarding endochondral growth at a time when bone is being substituted for cartilage. The interference caused the long bones to become thicker and shorter and also resulted in disproportionate bone formation around the central nervous system. In the skull epiphyseal growth on the base ceased, whereas appositional growth on the facial bones continued; pressure was thus exerted on the nervous tissues, as had been reported by Mellanby. Overcrowding of the spinal canal and cranial cavity resulted in the production of multiple herniations of the brain and a dislocation of it towards the foramen magnum.

Although severe vitamin A deficiency is rarely recognized amongst bovines in this country, we have not infrequently observed blindness due

* A draft of this paper was written by Professor Blakemore before his untimely death on 7th August 1955, and has been revised by the remaining collaborators as a tribute to his memory.

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to optic nerve constriction. The disease is similar in all respects to that described by Moore *et al.* (1935). In some cases it has been associated with a deprivation of vitamin A when blindness appeared. In other instances the current vitamin A status has been normal, although it has been evident that a previous deficiency existed. The disease is especially common on arable farms where straw is often substituted for hay and the diet includes a liberal allowance of sugar beet pulp.

In the present investigation calves were fed on a diet which had been observed to be responsible for blindness on affected farms so that its effects could be observed under experimental conditions. It was shown that alterations in vision occurred which could be traced to retinal abnormalities and changes in the optic nerves. The investigation afforded an opportunity to consider previous studies on other animals in relation to the development of ocular lesions in the bovine.

EXPERIMENTAL PROCEDURE

Five experimental bull calves and an equal number of controls were employed; all were Shorthorns with the exception of one Friesian which was included in the control group. They were reared normally, without access to pasture, and at the age of 3 months were castrated and housed for periods of 18 to 50 weeks in two groups in adjacent covered concrete yards littered with oat straw. The diet then consisted of sugar beet pulp, linseed cake and oat chaff, the amount allowed being calculated at the standard production rate according to age. Racks of oat straw were also provided.

The five control calves received a vitamin A supplement twice weekly in the form of vitamin A acetate, mixed with synthetic milk (Eden and Sellers, 1950) and given as a drench in doses calculated on the basis of a daily intake of 220 i.u. vitamin A per kg. body weight. To balance the small nutritional advantage of the controls, the experimental calves were dosed at the same time with a comparable amount of arachis oil made up in the same way.

The individuals in both groups were weighed weekly and their blood vitamin A values were estimated at frequent intervals. For this purpose the antimony trichloride method was used, and was applied by essentially the same technique as recommended by Kimble (1939). The average vitamin A levels for each group were recorded and liver vitamin A values were estimated on autopsy. The eyes were examined at weekly intervals and particular attention was given to the anterior and posterior halves of the ocular globes and adjacent structures. The results are summarized with the information relating to individual calves. The times of killing were chosen to correspond with varying stages of deficiency. A short time before the animals were killed cerebro-spinal fluid pressures were determined by means of a needle inserted in the dorsal opening of the atlanto-occipital articulation. The method described by Sykes and Moore (1942) was employed and the pressures were recorded in mm. of saline.

OBSERVATIONS

Growth. Throughout the experiments the calves in both groups continued to grow fairly steadily, but at widely divergent rates

between the individuals (Table 1). The number of animals was inadequate to decide whether growth was influenced by the presence or absence of vitamin A, but it was at least apparent that some of the undosed animals grew as well as those which were dosed.

TABLE 1

WEIGHT INCREASE OF CONTROL AND EXPERIMENTAL CALVES IN KILOS

No. of calf	*Experimental period in weeks*	*Weight (kg)*		*Increase (kg)*
		Initial	*Final*	
Experimental group				
1	18	45	62	17
2	31	55	150	95
3	37	57	111	54
4	47	71	340	269
5	49	50	174	124
Control group				
6	19	43	100	57
7	31	46	153	107
8	37	78	286	208
9	39	132	338	206
10	45	64	273	209

Vitamin A status. Immediately before the experiments started the levels of vitamin A in sera of the calves chosen for the experimental group were 28 to 58 i.u. (mean 42) per 100 ml. and in those chosen for the control group 43 to 90 i.u. (mean 59). Only low levels of carotenoids were present. These findings may be considered typical of young calves.

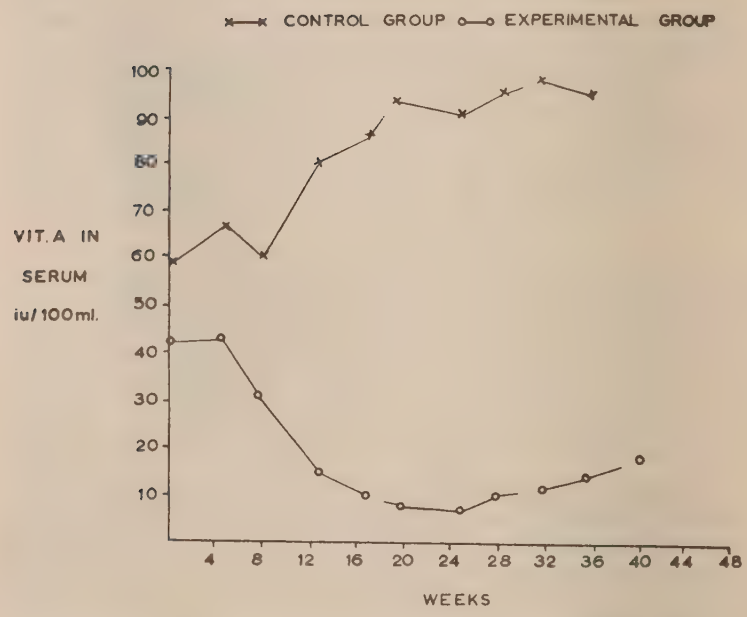
As the experiment proceeded the levels of vitamin A in the control group were raised and maintained above their initial values, usually at about 90 i.u. In the undosed calves, however, the levels first remained unchanged for 5 to 7 weeks, and then decreased, usually to 0 to 20 i.u. At these low levels accurate estimations were difficult. The average weekly levels of vitamin A for the two groups are shown in Fig. 1.

The liver reserves of vitamin A found at autopsy are given in Table 2. As expected, substantial reserves (25 to 230 i.u./g.) were found in the control animals, but the vitamin was absent, or present only in doubtful traces, in the experimental animals.

Deficient Calves

Progress of deficiency. At the point when the level of the vitamin A had decidedly decreased in the blood of the experimental calves, there were no clinical signs to indicate deficiency apart from some depression of appetite. At this early stage, however, ringworm developed in the experimental animals and spread to the controls.

FIG. 1



Average weekly levels of vitamin A

The disease resolved in the controls, but persisted in the deficient animals. It was associated with marked thickening and keratinisation of the dermis. After a period of 100 days the deficient calves were found to show definite signs of night blindness when put through a maze of straw bales at twilight. Soon afterwards it became apparent that the growth of the skull was not proceeding normally in the experimental animals. The disfiguration was manifested in the living animal as a doming of the frontal area. It was most marked in calf 5, although all the deficient animals were affected to some degree.

The following particulars relate to individual deficient calves.

Calf 1 was killed after an experimental period of 18 weeks. The weight increased 17 kg. Cerebrospinal fluid pressure was 125 mm. of saline. Growth was retarded and the weight of the calf remained almost constant

throughout the experimental period. Papilloedema appeared at 76 days and increased rapidly in intensity. At 101 days both globes were protruding and slightly displaced downwards. In both eyes the retina was excessively oedematous, but haemorrhage was not observed. Observations of the fundi became almost impossible due to rapid development of a superficial right corneal ulcer which developed into a large defect 6 × 13 mm. in size and later caused prolapse of the iris. This lesion subsequently healed and scar formation only was apparent at autopsy.

TABLE 2

LIVER RESERVES OF VITAMIN A AT AUTOPSY

No. of calf	*Experimental period in weeks*	*Weight of liver (g.)*	*Vitamin A in liver*	
			i.u./g.	*Total (i.u.)*
Experimental group				
1	18	1,300	<1·0	1,300
2	31	2,850	1·1	3,200
3	37	2,170	<1·5	3,300
4	47	2,550	0·0	0
5	49	—	0·1	—
Control group				
6	19	1,800	25	45,000
7	31	2,480	230	570,000
8	37	3,600	110	400,000
9	39	5,000	121	610,000
10	45	5,300	145	770,000

Control group. Each animal received 220 i.u. of Vitamin A acetate/kg. body weight/day.

Calf 2 was observed over an experimental period of 31 weeks. The weight increased by 95 kg. The cerebrospinal fluid pressure was 125 mm. The growth rate was satisfactory, but towards the end of the experiment the animal was not in good condition and there was marked doming of the head. Papilloedema appeared at 76 days and at 96 days the globes began to protrude in an anterior and downward direction. Papilloedema developed to its maximum intensity at 118 days. The retinal veins were full and bent over the edges of the oedematous alley with numerous haemorrhages on and near the discs. This picture remained the same until total blindness developed suddenly at 135 days. It was then observed that the pupils were dilated and showed no reaction to light. At 152 days the papilloedema began to spread. Atrophic changes were observed in the retina when the animal was killed at 217 days.

Calf 3 was observed over an experimental period of 37 weeks. The weight increased by 54 kg. The cerebrospinal fluid pressure was 150 mm. This animal did not thrive and at an early stage showed nervous symptoms following any undue exertion or excitement. During these attacks, which lasted 1 to 2 minutes, it appeared to faint, lost control of its limbs and lay with neck and legs extended. There was no spasm and the muscles were flaccid. These attacks continued until it was killed. There was an ocular

disturbance at 120 days, evidenced by bilateral corneal infiltration and excessive lacrimation which responded to treatment with penicillin, leaving a corneal scar. Examination of the fundi was difficult, but papilloedema was recognized at 130 days associated with retinal haemorrhage.

Calf 4 was observed over an experimental period of 47 weeks. The weight increased by 269 kg. The cerebrospinal fluid pressure was not determined. Although this animal obviously became deficient it grew more rapidly than any of the controls. Doming of the skull was not prominent and the eye changes were relatively slight. At 106 days there was some displacement of the left globe which gradually became more prominent. Slight oedema of the right retina was observed at 96 days and the left soon became affected, but the changes developed slowly in comparison with the other animals. Retinal haemorrhage and exudation appeared at 223 days when papilloedema reached its maximum intensity.

Calf 5 was observed over an experimental period of 49 weeks. The weight increased by 124 kg. The cerebrospinal fluid pressure was 80 mm. The development of this calf was similar to calf 2. Papilloedema was noted in the left eye at 131 days and soon became pronounced in both eyes. It was associated with numerous haemorrhages of the retina, many of which were quickly absorbed and were followed by fresh ones.

Control Calves

The five calves remained normal and individual animals were killed at approximately the same time as the experimental calves, so that a comparison could be made between the two groups throughout the experiment.

Their eyes were subjected to the same examinations as the experimental group and no significant retinal abnormalities were found. They all showed symptoms of a mild conjunctivitis, however, for a short period and in 10 this was followed by a superficial corneal ulcer which healed after 14 days. The cause of this conjunctivitis was not ascertained. Cerebrospinal fluid pressures were determined immediately before they were killed and varied from 75 to 95 mm. The procedure at post-mortem examination was the same as for the experimental group and no significant gross or microscopic abnormalities were found.

Post-mortem Findings in the Deficient Calves

Calf 1 showed lesions indicative of a previous broncho-pneumonia affecting the apical and cardiac lobes. There were no gross abnormalities in the viscera of the remaining calves. The brain, skull and eyes of each calf were carefully examined. Sections of the lungs, trachea, liver, kidney, bladder and intestinal tract were also studied for evidence of epithelial abnormality. Although these were not found to be either striking or consistent, marked hyperplasia was detected in the bronchi of 1, 2 and 3, and in the bladder of 2. Renal sclerosis of the kind often found following coliform infection was also observed in 5. The absence of marked squamous metaplasia in the vitamin A deficient calves in the tissues examined is in accordance with the

findings of Jungherr, Helmboldt and Eaton (1950) who found in the bovine that significant changes were consistently present only in the epithelial lining of the parotid gland.

Skull. The doming of the head, which affected all the experimental calves to a varying degree, was traced to a convexity of the parietal and frontal bones. Longitudinal sections of the skulls in the sagittal plane revealed a "packed-in" appearance of the brain, which was especially pronounced in 2 and 3; the latter also showed a thickened frontal bone. The substance of the bones on the base of the skull showed varying degrees of change, particularly at their epiphyses; thus the long axis of the post-sphenoid was more oblique and the pre-sphenoid elevated in position. As was noted later when the brain was removed, this affected the manner of articulation between the wing of the sphenoid and the frontal bone — it could easily have effected kinking of the nerve. The basi-occipital and post-sphenoid bones were relatively thin and the pituitary fossa deep.

Brain. In the experimental calves the brain was compressed and the conformation of the various parts was altered. Although there were slight differences between individual specimens the common effect produced resulted in the line of the medulla-pons-cerebral peduncles forming a more upward curve than usual, which exaggerated the downward direction of the cerebrum and olfactory areas rostral to the pituitary. Thus, the arching of the mid-brain area was associated with bony changes on the base of the skull, and this in turn was in keeping with the curvature formed by doming of the frontal bone area.

The cerebellum and cerebrum were more approximate and showed compression changes, particularly in the dorso-ventral direction. In some cases the medulla showed pressure from the cerebellum and in others the corpora quadrigemina showed pressure from the cerebrum. The state of the ventricle aqueducts suggested an increase of cerebrospinal fluid. The optic tracts and chiasma were subjected to pressure between brain and bone, and this was most marked in 3.

Orbit. Following examination of the brain in situ, the skull was sectioned transversely at a level mid-way through the orbital rim, the depth of the cut extending through the ethmoid bone capping the olfactory lobe. The optic nerve, still attached to the eye, was then exposed by dissection of the muscles. When the retractor muscle surrounding the nerve was cut it was found in 3, 4 and 5 that the slight curvature normally found in the portion of the nerve approximate to the eye was grossly exaggerated (Figs. 2 and 3). (The altered disposition is shown in situ in Fig. 4). In 1 it was so changed that the nerve formed an S-shaped curve. The distorted nerves were longer than normal, as is consistently found in the natural disease. There was also evidence of twisting of the nerve on its long axis, which persisted when the nerve was removed from the orbit (see Figs. 2

and 3). In calf 2, the blind animal, each optic nerve was fixed to the muscle as it emerged from the optic foramen, and was surrounded by a mass of fibrous tissue adherent to both bone and nerve. Included within this tissue were extrusions of the meningeal sheath. The size of the optic foramen and canal was determined in both groups of calves by insertion of a probe of graduated diameter. No significant difference was found between the two groups (Table 3).

TABLE 3

WIDTH OF OPTIC FORAMEN AND CANAL

No. of calf	*Width of optic foramen*		*Width of optic canal (mm.)*
	At base of orbit (mm.)	*At chiasma (mm.)*	
Experimental group			
1	5·0	5·0	11
2	4·0	4·0	13
3	5·5	5·25	11
4	5·25	5·0	18
5	4·5	4·25	13
Average	4·8	4·7	13
Control group			
6	4·0	4·75	7
7	5·5	5·0	17
8	5·25	5·4	12
9	5·5	5·0	18
10	5·5	5·7	15
Average	5·2	5·2	13

The optic nerve is peculiar in that it is surrounded by an outer pigmented sheath derived from the dura mater, i.e. it is an extension of the meningeal sheath, and this covering contains many small capillary blood vessels and nerve fibres. It is in the sheath that the first reaction of the affected nerve appears to take place. In 3 this was evidenced by increased thickness and vascularity without any change in the nerve fibres. In 2 the nerve was extensively affected and sections showed a reaction of the sheath and neurilemma associated with an increase in vascularity and extensive cellular and connective tissue infiltration of the nerve, resulting in the isolation of islands of degenerate and vacuolated fibres (Fig. 5 *a* and *b*). The connective tissue between the fibres appeared to arise from both the sheath and neurilemma, which formed an adherent mass. This proliferation from the sheath into the nerve is also observed in the field cases of the disease. In fact, in some instances, it may be so marked that the nerve becomes obliterated at the foramen and is divided into two segments, each being capped by the sheath and neurilemma, and joined together by loose connective tissue. A

typical example of the effect produced in a field case is depicted in Fig. 6.

Pituitary. The pituitaries of three of the experimental calves (3, 4 and 5) showed well-marked cysts. In each case the cysts were situated between the intermediate and anterior zones, in the vestigial cleft of Rathke's pouch, and it was noted that the cavity of the cleft and the cavity of the cyst were continuous. There were no changes in the tissue of the two zones indicative of pressure atrophy. The infundibular recess of the pituitary, an extension of the third ventricle of the brain, extends into the pituitary for only a short distance in cattle; in these experimental animals its lumen was not associated with that of the cyst.

DISCUSSION

It has already been shown by other workers that impairment of vision involving the optic nerve occurs in young cattle on Vitamin A deficient diets. The object of this investigation was to ascertain whether a diet of sugar beet pulp, straw and a protein cake, which is not infrequently used for young stock on arable farms in this country, would produce this lesion in calves under experimental conditions. The results indicate that a serious dietary deficiency does occur which may cause blindness.

Although the deficient calves did not appear to be in good condition, only three showed a considerably lower weight increase as compared with the controls. In two of these animals the defective growth could be attributed to complications arising from the deficiency, for one suffered from epileptiform convulsions and another was affected with broncho-pneumonia. One, which became blind, grew normally. Although only a small number of animals was examined, the results indicate that it does not necessarily follow that deficient animals are stunted or undersized. In the natural disease blindness may be the only symptomatic abnormality and this is in accordance with our findings.

With the exception of one calf, no significant visceral abnormalities occurred in the deficient calves. Pneumonia in this animal was associated with pronounced hyperplasia of the bronchial epithelium which may possibly have been responsible for areas of pulmonary collapse in which infection had become established. The same animal also suffered from corneal ulceration which healed despite the persistent deficiency. Mild conjunctivitis also appeared in the controls. Although the aetiology was not established the condition responded to treatment and was probably due to an intercurrent infection. Histological examination failed to show that metaplasia and keratinisation of the epithelial surface are a pronounced or consistent feature of vitamin A deficiency in cattle. In this respect our findings confirm those of Jungherr *et. al.* (1950).

There were no gross changes in the bones other than those of the

skull, where disproportionate bone growth resulted in a doming of the frontal area and distortion of bones on the base of the skull. Such findings could be attributed to almost complete cessation of endochondral growth and reduction of appositional growth, resulting in growth pressures at either extremity of the cranial cavity, with the contained nervous tissue growing unimpeded. In this respect the sequence of events is in agreement with the findings of Wolbach (1947), who observed that continued growth of nerve after cessation of bone growth led to constriction of the cranial cavity, multiple herniations and dislocation of the brain towards the foramen magnum. These abnormalities were confirmed in the experimental calves. The increased length of the optic nerve within the orbit, evidenced by kinking and twisting, can also be seen in this sense, in that growth of the optic nerve is not accompanied by a comparable growth of bones forming the optic wall.

There are difficulties in assessing with accuracy the cerebrospinal fluid pressures in bovines, but the anatomical findings were indicative of increased values and supported the ante-mortem determinations. The papilloedema could be attributed to increased cerebrospinal fluid, and it is known that in adult cattle papilloedema may be the sole factor causing temporary blindness (Moore, 1941).

The occurrence of pituitary cysts in the experimental calves calls for special consideration since so-called cysts have been recorded in normal cattle. For instance, Bassett and McMeekan (1951) recorded their presence, without pressure atrophy or cellular changes, in the glandular tissue. Bassett (1951) regards colloid as a normal constituent of the mature pituitary and states that it may be found in the cleft, in blood vessels and in the anterior lobe. We have examined a random sample of 125 glands collected from a local abattoir and our findings were in general agreement with this conclusion. It is possible, however, that these so-called cysts may represent an exaggeration of the normal tendency and may lead to pressure atrophy and cellular change. The occurrence of cysts in four of the five experimental calves may indicate that vitamin A deficiency is a factor in their causation, as claimed by some other workers (Madsen, Hall and Converse, 1942; Helmboldt, Jungherr, Eaton and Moore, 1953). Distension of the pituitary was also observed by Mellanby (1939) in dogs deficient in vitamin A. In this animal the abnormality could be produced by an increase in the cerebrospinal fluid pressure because the infundibular cavity is prolonged into the pars nervosa. Although there are anatomical differences in the ox, since the infundibular cavity is only slightly prolonged into the pars nervosa, it is possible that this encroachment may be exaggerated in vitamin A deficiency, leading to pressure changes. Furthermore, displacement and bone pressure are other factors which may affect the gland.

The actual cause of the constriction of the optic nerve has been assumed by some workers to be a failure of the foramen and optic canal to re-model during growth so that they can accommodate the

nerve as it increases in size. Reference to Table 3 indicates that in the experimental group there was little alteration in diameter of the foramen or canal and yet there were marked changes in the nerve; in one calf in which the optic nerve became completely obliterated there was no alteration in the diameter of the foramen. Constriction can therefore result from other causes. These include abnormal shape of the foramen resulting from different rates of individual bone growth (Crocker, 1919), a tendency for the nerve to twist in the canal and orbit, changed alignment of the bones associated with the course of the optic nerve from the chiasma to the eyeball and increased cerebrospinal fluid pressure. Moreover, even when bone growth is complete, a form of temporary blindness with associated papilloedema may occur (Moore, 1941). The rapid onset of total blindness, which is a feature of many cases of the disease, suggests that it may, on occasions, result from sudden twisting of the nerve at the optic foramen.

The response of the nerve following constriction is first evidenced by a reaction of the dura mater lining the foramen. The resulting fibrosis causes pressure on the nerve fibres and finally invades the nerve to such an extent that it may sometimes completely divide it into two parts, each encapsulated by proliferated sheath.

CONCLUSIONS

Blindness may occur when calves are reared under natural conditions on a diet of sugar beet pulp, straw and protein cake. The effect of this diet was studied experimentally in five calves. Five control calves were fed in the same way, but with supplements of vitamin A acetate twice weekly.

Night blindness and abnormalities in vision occurred in the deficient calves. They developed papilloedema and one became totally blind. The growth of the bones of the skull was disproportionate, leading to constriction of the brain and increased cerebrospinal fluid pressure. Marked constriction of the optic nerve was found in the blind animal. This was not associated with a narrowing of the optic foramen, but appeared to be due to a number of factors including malformation of the foramen, increased cerebrospinal fluid pressure and a tendency for the optic nerve, which was increased in length, to twist at the site of the foramen. The changes are discussed in relation to the natural disease. Reference is made to pituitary cysts.

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LEGENDS TO ILLUSTRATIONS

Fig. 2. Section of brain, optic nerve and eye to show exaggerated curvature of optic nerve.

Fig. 3. As Fig. 2 with muscles reflected.

Fig. 4. Section of optic nerve and eye in situ (left) to show exaggerated curvature of the optic nerve. (The accompanying photograph is from a control animal).

Fig. 5 *a* and *b*. Sections of optic nerve showing cellular and fibrotic change.

Fig. 6. Section of optic nerve from a field case showing complete division.

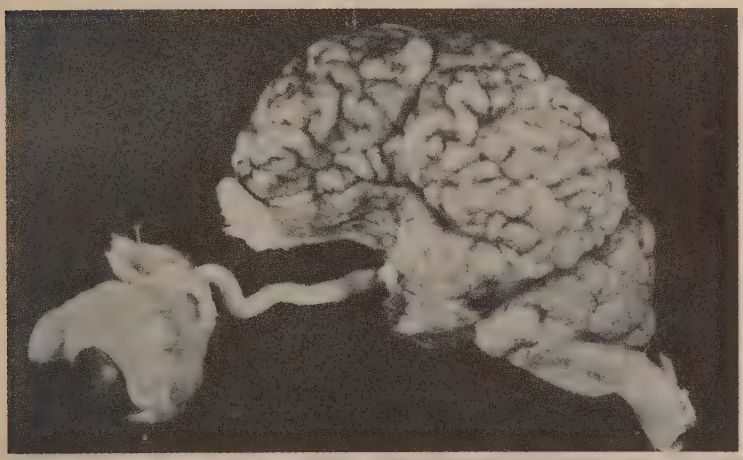

Fig. 2

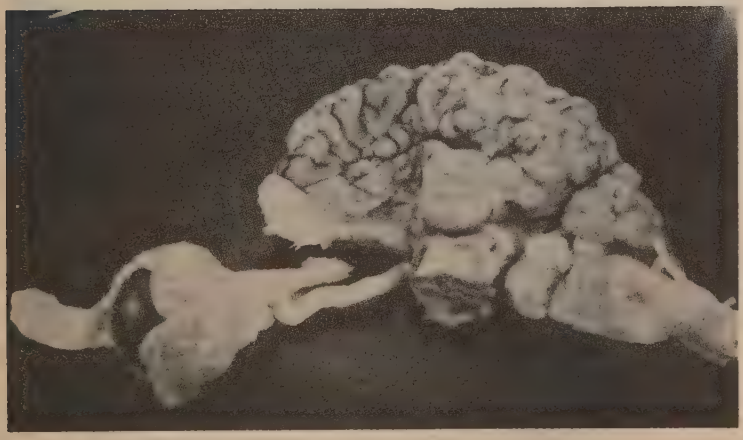

Fig. 3

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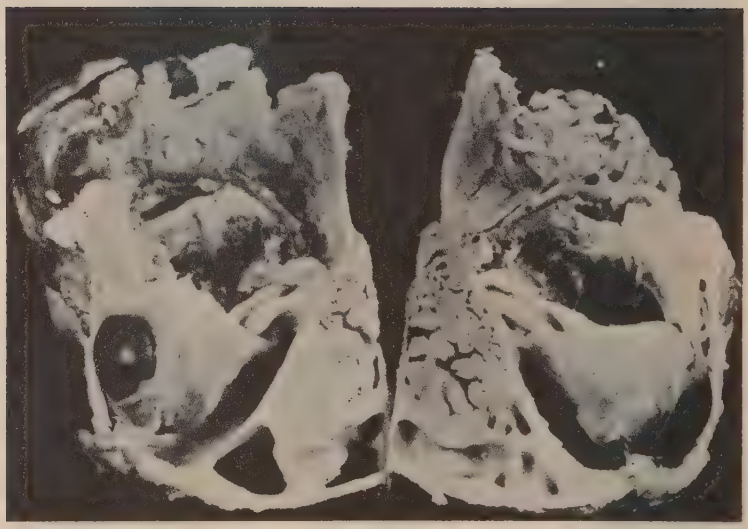

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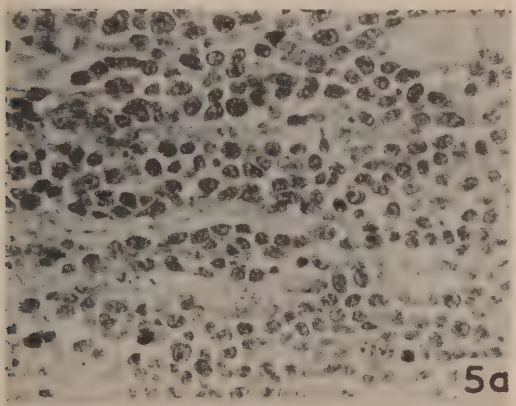

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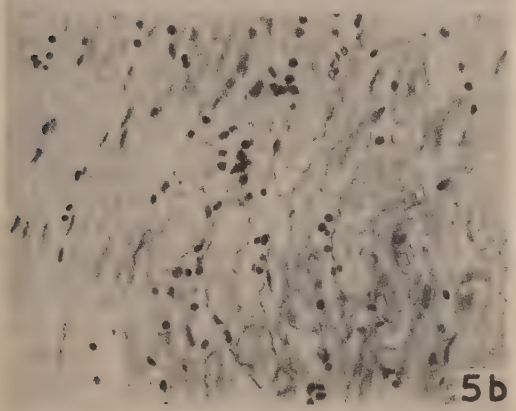

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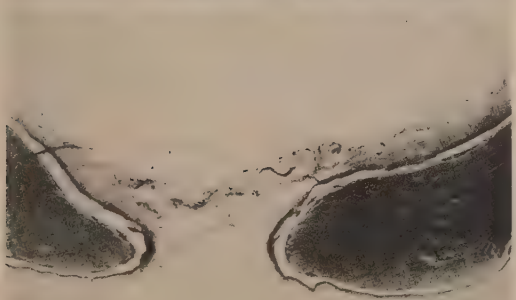

6

THE CONCENTRATION OF BLOOD SUGAR DURING STARVATION IN THE NEW-BORN CALF AND FOAL

By

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INTRODUCTION

Graham, Sampson and Hester (1941) observed that hypoglycaemia was a pathognomonic feature in a fatal disease of new-born pigs and subsequently made the important observation that starvation alone could rapidly produce a similar syndrome (Sampson, Hester and Graham, 1942). In contrast, Hanawalt and Sampson (1947a) found that older pigs, weighing between 20 and 40 lb., were resistant to a long period of starvation (24 and 28 days), the main development of this resistance occurring during the first week of life (Hanawalt and Sampson, 1947b). Thus starvation from birth was fatal in less than 30 hours, whereas if the pigs were 7 days old when first starved, death did not follow until nearly 2 weeks later. The survival time of pigs that are removed from the sow at birth, however, is subject to considerable variation and it has been shown that the ambient temperature is one of the major determinants in this respect (Morrill, 1952). Goodwin (1955) believed that hypoglycaemia was a non-specific sign in the pathology of the new-born pig and that, like azotaemia, it represented a common end-point in the pathogenesis of many diseases. Hence there are both clinical and fundamental reasons for observing the effect of starvation on the new-born of other farm animals. Sampson, Taylor and Smith (1955) found that the new-born lamb does not develop hypoglycaemia so rapidly as the new-born pig when starved and this paper presents similar data for the new-born calf and foal. In addition to observing the stability of the blood-sugar concentration and the general clinical signs during starvation in these last two species, the present experiments have provided information on the blood-glucose level during the first hours of life.

MATERIALS AND METHODS

Animals and experimental procedure. Observations were made on nine calves and one foal. Calf 8, which was a Jersey, was included as the remaining calves were all Ayrshires and it was possible that the resistance might vary between breeds. The foal was from a New Forest × Shetland mating. Parturition was attended and none of the animals was allowed to suckle before being removed to the experimental accommodation. No water was provided during starvation, since under natural conditions, failure to suckle deprives the new-born of both food and water. The foal, however, received water after 7 days, when it was apparent that dehydration could become the limiting factor in survival. Protective serum was not given at first, but, for reasons that are mentioned later, this proved

unsatisfactory; accordingly calves 5 to 9 and the foal received between 100 and 300 ml. of maternal serum shortly after birth. With calves 1, 2, 3 and 5, the environmental temperature during starvation was recorded on a maximum/minimum thermometer, but as the maximum temperature recorded was 52°F., at which temperature the starving new-born pig succumbs rapidly, further detailed records were not taken.

Estimation of blood-sugar concentration. Blood was taken from the jugular vein and collected over dried heparin. Duplicate and, occasionally, triplicate samples of 2 ml. each were then deproteinized with barium hydroxide and zinc sulphate, and the filtrates were analysed for their sugar content using the methods outlined previously (Goodwin, 1956a). The cooling technique for estimating the concentration of sugar in the plasma has also been described elsewhere (Goodwin, 1956b). Whenever the estimations were of glucose alone, a glucose standard in quadruplicate was included with the unknowns, and when the filtrates contained both fructose and glucose, mixed standards usually equivalent to three declining levels of fructose in the presence of an appropriate physiological concentration of glucose were estimated in duplicate. Any errors occurring in the standard estimations were allowed for by adjusting the final values of the unknowns.

RESULTS

The Calf

Blood-sugar concentration. The variations in blood-sugar concentration for the nine calves are depicted in Fig. 1. An arbitrary division into two groups has been made in order to reduce the number of overlapping graphs and the first 30 hours of life are represented by a different time scale. The fructose present at birth rapidly leaves the circulation and by about 15 hours the blood sugar can be taken as glucose. With the exception of calf 8, the blood-glucose concentration was at first between 75 and 125 mg. per 100 ml. If not already high, the concentration then tended to rise to a plateau of between 100 and 130 mg. per 100 ml. Between about 20 and 30 hours after birth the glucose concentration began to decline to a lower level, which was then maintained fairly constantly. The limits of this final concentration were approximately 55 and 85 mg. per 100 ml. with most values lying between 60 and 80 mg. per 100 ml. The low terminal values for calves 1 and 8 will be considered later with the clinical observations. In calves 3 and 6 the blood-glucose concentration of starvation was interrupted by a comparatively sudden upsurge to a high level of well over 200 mg. per 100 ml. This phenomenon may be accepted as real in calf 3 because the peak value was followed by a succession of declining values; furthermore in calf 2, which was born within a few minutes of calf 3 and starved in the same box, there was a coincident, although less severe, increase in glucose concentration. The same apparent lack of control of the glucose concentration is even more convincing in calf 6, as there was at first a violent fluctuation in sugar concentration and then a slowly mounting level before the sudden peak of nearly 250 mg.

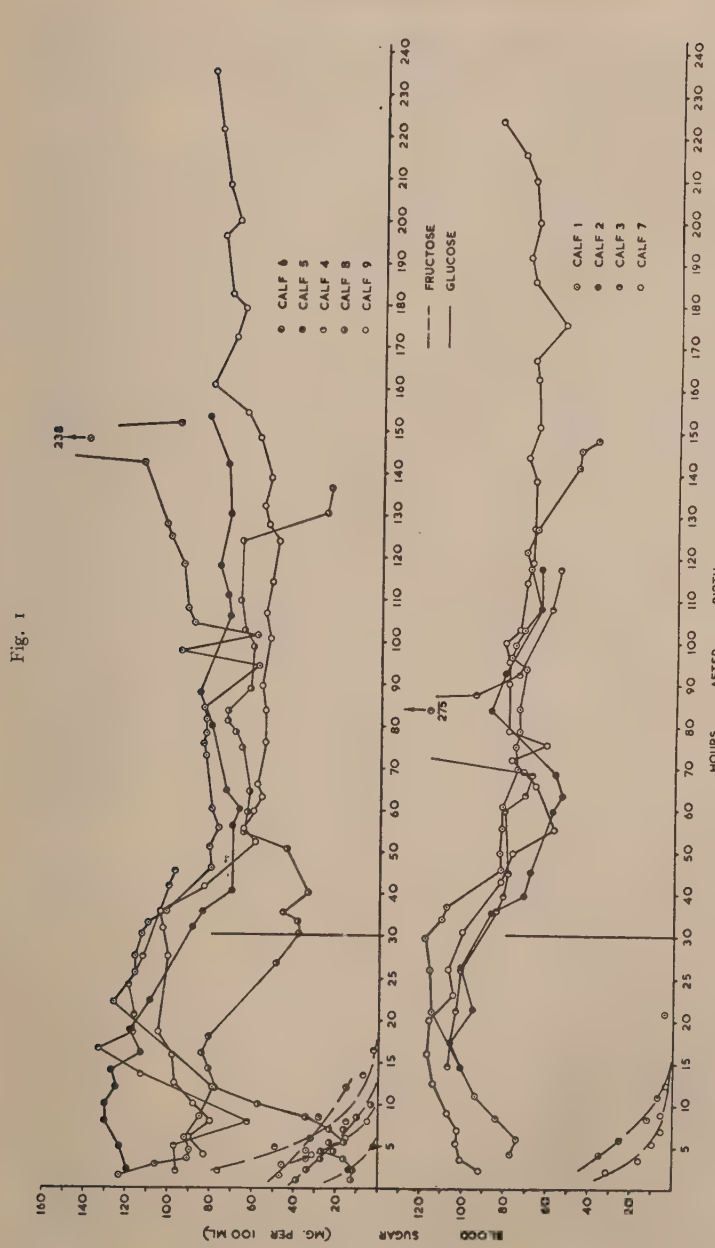

Fig. 1

Concentration of blood sugar in the starving new-born calf.

per 100 ml. was reached. Owing to the infrequency of sampling, the maximum glucose concentration that was reached in these circumstances is not known; nor is the duration of marked hyperglycaemia known, but it could have been brief.

Clinical observations. Three calves (1, 4 and 8) departed materially from the general pattern and they will be described first. Calf 1 remained strong until the day before its death. After 6 days of starvation, however, it suddenly became very weak and cold. As its condition did not respond to glucose therapy it was shot. No post-mortem examination was made and it seemed likely that the animal had succumbed to hypoglycaemia. Calf 4 also died suddenly, being very strong at 45 hours and dead at 62 hours, but the early death of this animal could not be so readily ascribed to hypoglycaemia and a post-mortem examination revealed the presence of septicaemic changes, particularly cardiac haemorrhages, while *Bacterium coli* was cultured from the heart blood, spleen and liver. In the light of these findings, together with the fact that calf 3 had developed a swollen carpal joint that responded to Streptomycin, it must be concluded that calf 1 probably died from septicaemia also. The risk inherent in keeping calves colostrum-free was obviated in the remaining animals (5 to 9) by injecting them subcutaneously with serum, and no further difficulties of the above type were met. The Jersey calf (8) never appeared completely normal; it was weak at birth and was not seen to get to its feet unaided until the 64th hour. At no time was it energetic and it was found dead after $5\frac{1}{2}$ days. A post-mortem examination did not implicate any infective organism. It can be seen that the blood-glucose concentration in this animal was grossly abnormal shortly after birth. Although eventually the glucose level remained within the range shown by the Ayrshire calves, the ultimate collapse could well be a reflection of the weakness in the glucose regulation of this particular animal rather than an indication of increased susceptibility among Jersey calves generally.

Apart from the three atypical calves above, starvation had little obvious ill-effect and there were no signs indicative of distress. Thus calves 7 and 9 were walking well after 10 days of starvation and calves 2, 3, 5 and 6, which were without food for an average period of 6 days, would obviously have withstood further deprivation. The most usual behaviour was for the calf to be robust for 4 or 5 days, jumping to its feet when approached, and thereafter to become less interested in sucking or other activities. The rectal temperatures decreased only slightly, the maximum decrease in calves 1, 2 and 5 being 2°, 1° and 2·5°F., respectively, and with calf 3 there was no decrease. The heart rate, however, decreased considerably: in calves 1, 2, 3, 5 and 8, all starved for 5 to 6 days, the rate decreased to between 53 and 73 per cent of its original value, while in the two calves starved for 10 days the corresponding figures were 60 and 71 per cent. Part of this decrease was probably due to the fact that the animals gradually became indifferent to constant examination. There was

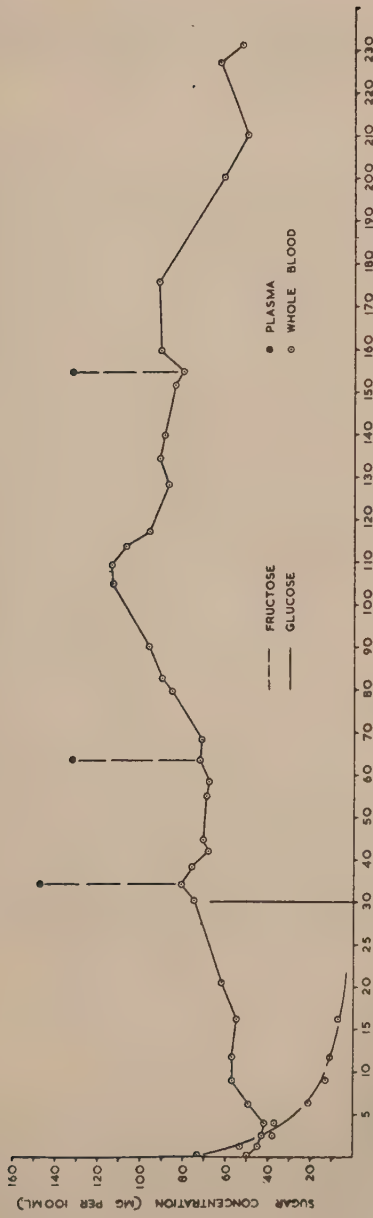

Fig. 2

Concentration of blood sugar in a starving new-born foal.

also a marked loss of weight : calves 2 and 3 changed from 67 and 77½ lb. to 56½ and 67 lb., respectively, after nearly 5 days of starvation, while the longer starvation (10 days) of calf 9, which weighed 91 lb. originally, produced a weight loss of 19 lb.

The Foal

Blood-sugar concentration. Conclusions derived from Fig. 2 must necessarily be limited by the fact that only one animal was studied. As with Fig. 1, the time scale before 30 hours is exaggerated. The decline in fructose concentration accords with expectation and need not be considered further. The blood-glucose concentration increased progressively for 5 days, fell slightly and was then maintained at the high level of about 90 mg. per 100 ml. for a further 3 days. Before making comparisons with the calf, however, it is important to take into account the difference that exists between the calf and foal in the distribution of sugar between the red cells and plasma (Goodwin, 1956b). Whereas the erythrocyte of the calf contains a high concentration of glucose, in the foal the glucose is largely confined to the plasma, as can be seen from the three values for plasma-glucose concentration in Fig. 2. Thus the plasma-glucose concentration in the foal is considerably greater than in the calf at the same whole-blood glucose concentration. In this particular foal, therefore, the plasma-glucose concentration was maintained during the main period of starvation at a much higher level than would have been shown by the calves. Furthermore, the patterns shown by the changing concentration of glucose with time differed in the two species, for the foal did not show, during the first day of life, the relatively high glucose concentration that was common in the calf.

Clinical observations. The animal was standing strongly within an hour of birth and remained almost normal clinically for 4 days. Subsequently, it showed evidence of dehydration and by the seventh day some weakness was apparent. Thereafter, water was given *ad lib.*, and this had a remarkable revitalizing effect. Although subdued for the remainder of starvation, it was still walking voluntarily after 10 days and it remained inquisitive about its surroundings. Despite the intake of about 14 pints of water during the last 3 days, the body-weight decreased from 70 to 55 lb. During the first 7 days the rectal temperature showed a maximum fluctuation of 2°F. and the heart rate decreased from 88 to a minimum of 56 beats per minute.

DISCUSSION

It is clear that the new-born calf and foal, unlike the new-born pig, are not readily susceptible to hypoglycaemia during starvation, as at the environmental temperatures of the present experiments the new-born pig would have survived for less than 2 days. Although no attempt was made to determine the limits of endurance for the calf and foal, it would seem that they are probably more refractory to

starvation than the lamb; for Sampson *et. al.* (1955) found that lambs that were already one-day old became comatose in about 8 days when the environmental temperature was approximately 70°F. It is interesting that the new-born calf and foal should show such resistance even though they were denied water, and the resistance to hypoglycaemia might be enhanced if water were taken from birth in the quantities that are normally suckled from the dam. The main clinical significance of these findings is that disease processes in the new-born calf and foal can continue longer and invoke more characteristic clinical syndromes than in the new-born pig where the vulnerability to hypoglycaemia imposes a common clinical mould on much of the known neonatal pathology and inhibits the maintenance of signs as fundamental as pyrexia and tachycardia.

The blood-sugar concentration in the starving new-born calf appears to be regulated at two levels: first a high initial level of over 100 mg. per 100 ml. and then a lower level of almost half this concentration, the transition taking place during the second day of life. It is probable that the calf, like the pig, is born with a high concentration of glycogen in the liver and that the blood-glucose concentration shortly after birth can be maintained at an optimum level. During the second day, when without gluconeogenesis the glycogen supply would be expected to fail, the glycogen and glucose available to the circulation would be limited by the rate of gluconeogenesis, and this seemingly compels a lower sugar concentration. The latter may be compared with the average post-prandial value of nearly 100 mg. per 100 ml. obtained by McCandless and Dye (1950) in fed calves during the first 10 days after birth. An alternative explanation of the higher glucose level during the first day is possible. It has been observed that when the young pig is suffering from respiratory or circulatory embarrassment after birth there is often a hyperglycaemic response which persists for a few hours and subsides with the recovery of the animal. It seems unlikely, however, that most calves would suffer such embarrassment immediately after birth and that the return to normality should occupy up to about 30 hours. No explanation can as yet be given for the regulatory failure that resulted in the temporary but severe hyperglycaemia in calves 3 and 6.

CONCLUSIONS

Eight Ayrshire calves were starved from birth for between 5 and 10 days. Two out of four calves that received no maternal serum died. The remaining calves, which were injected with serum, withstood starvation well and they were reared without difficulty. A single Jersey calf was also starved from birth. Although born weak, it survived for 5½ days. A pony foal was starved for 10 days and survived.

In the calf starved from birth, the blood-glucose concentration generally reached about 100 to 130 mg. per 100 ml. during the first 30 hours. Thereafter, in the animals that survived, it declined to

between about 55 and 85 mg. per 100 ml. for the remainder of starvation. If plasma-glucose concentrations are compared, it is possible that under similar conditions the foal maintains a higher circulating glucose concentration than the calf.

ACKNOWLEDGMENTS

The post-mortem examinations were made by Mr. I. D. B. Newsam. The author is indebted to Mr. R. E. D. Hansen for attending all the calvings and isolating the calves, and to Mr. P. J. D. V. Brett for general laboratory assistance. Most of the expenses were met by a grant from the Agricultural Research Council.

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THE STUDY OF SOME VIRUSES BY THE PLATE GEL DIFFUSION PRECIPITIN TEST

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INTRODUCTION

The gel diffusion precipitin test of Ouchterlony (1948) has stimulated the study of viral antigen-antibody reactions by this method. Jensen and Francis (1953) reported the presence of a specific reaction with influenza virus. Molnar (1954) demonstrated a reaction between swine fever antigen and immune serum, and Boden (1955) typed foot and mouth disease virus. Gispen (1955) distinguished between vaccinia and cow pox: he also studied the diffusion pattern of ectromelia, rabbit pox and fowl pox. Modifications in the technique of the test were reported by Wilson and Pringle (1954) and by Feinberg (1956).

In this paper observations are made on a further modification in the technique described by Bjorklund (1952) in the study of the following virus diseases:— myxoma (MYX), fibroma (FIB) of rabbits, canine distemper (DCX), Rubarth's disease (HCC) in dogs, lymphocytic chorio-meningitis (LCM) and swine fever (S.F.).

MATERIALS AND METHODS

Medium. To make one litre of medium the following ingredients were used:— 15 g. agar (Davis powdered agar — New Zealand), 16 g. sodium chloride (Hopkins and Williams — England), 50 ml. of 10 per cent. phenol saline solution, 0·03 g. methyl orange dissolved in distilled water. The agar was melted and other ingredients mixed and filtered through paper pulp. A standard volume of 25ml. of medium was poured into each Petri dish specially chosen to be free from scratches or cloudiness. The plates were allowed to cool until the medium solidified.

Basins. Each plate was stamped four times with a pattern consisting of a central basin surrounded by six other basins to form a hexagon. These basins were cut out from the medium with an appliance which consisted of four sets of cutting units. Each unit comprised seven steel tubes, a perspex disc, a brass disc and a rubber bung (Fig. 6). The metal tubes were cut to a length of 6·5 cm. long and were arranged so that six were around and equidistant from the central seventh tube. The distance between the tubes was 0·4 cm. and each tube had a diameter of 0·7 cm. The tubes were held in place by perspex and brass discs and were fixed to the latter. The ends opposite the cutting ends of the apparatus were then passed through a drilled rubber bung which was attached to the vacuum flask and pump. Four units were fixed in one apparatus and it thus needed only a single process to produce a symmetrically arranged pattern. Each plate when being cut was held upside down to prevent any dust settling on the surface of the media and also to help suction. The bottom of each basin was sealed with a drop of melted medium. To avoid

scratching or damaging the glass, plates were labelled with type-written zinc oxide tape, the label being placed on the rim of the part of the plate containing the medium.

Antigens. The antigens, in the form of tissue suspensions or small pieces of unprepared tissue, were placed in the basins and covered with one drop of 0·5 per cent. phenol saline.

Sera. Sera were used undiluted and unheated.

Readings. Plates were either left at room temperature or incubated at 37°C. for twenty-four hours before readings were made. An indirect light from a powerful microscope lamp reflected by a concave mirror aided examination of the plates. The peripheral basins of the hexagonal pattern were recorded as Nos. 1, 2, 3, 4, 5, and 6 in a clockwise direction, the top basin in the circle being No. 1.

RESULTS

Determination of the Suitable Distance between Antigen and Antibody Basins

As shown in Fig. 1, myxoma antigen, in the form of small pieces of infected oedematous lesions, was placed in the central basins. Normal rabbit serum was placed in the right hand side basins and myxoma immune serum in those on the left hand side. The distances between antigen and sera were 0·2, 0·4, 0·6, 0·8, 1·0 and 1·6 cm. on either side of the antigen. A precipitin reaction occurred after two and a half hours when the distance between the basins was 0·2 cm. After four hours the reaction appeared in the basins 0·4 cm. apart, and after being left overnight on the bench the reactions appeared at a distance of 0·6 and 0·8 cm. At a distance of 1·0 cm. a faint reaction was seen at about thirty-six hours. The reaction had appeared at a distance of 1·6 cm. by ninety-six hours, results shown in Fig. 1 having occurred forty-eight hours after the plate had been prepared.

From this experiment it was clear that the distance between basins was of importance in reading the test. The sharpness of the reaction also varied: when the distance was as narrow as 0·2 cm. the reaction was strong and blurred, when the distance was 0·8 cm. or more the lines were faint. A distance of 0·4 cm. was the optimum for the antigen-antibody systems in myxoma, fibroma, swine fever, canine distemper, Rubarth's disease and lymphocytic choriomeningitis, and was used throughout this work.

The Difference between the Use of Tissue Suspensions and Untreated Whole Tissues

In earlier studies using swine fever and canine distemper infected tissue suspensions, the reactions were either very faint or absent. It was, therefore, decided to use the whole untreated tissue in the form of a small piece placed in the basin. Care was taken to fill the basin so that tissue should come into contact with the entire edge. The reaction was markedly increased. The precipitin line or lines produced were sharp and distinct. This method minimised the labour

involved in grinding and the time taken in making the suspensions and in cleaning and sterilising the grinders. It was also economical in the use of material.

Disappearance of the Virus-antigen in the Tissue after Exposure to Immune Serum

A piece of DCX infected tissue was placed in a basin and exposed to DCX serum in the opposite basin. The precipitin reaction occurred after twenty-four hours. The same tissue was removed and placed in a fresh plate and exposed once more to DCX serum. No reaction occurred.

Resistance of the Precipitin Antigen to Freezing and Thawing

An experiment was made, using swine fever infected tissue to determine the resistance of the precipitin antigen to freezing and thawing. A piece of tissue was placed in deep freeze at —70°C. overnight, and was then thawed at room temperature: this was repeated twelve times. A small sample was tested after each thawing and no difference was observed in the nature of the line produced when exposed to swine fever serum. When a similar piece of tissue was placed at —70°C. and then thawed quickly in a water bath at 37°C., no reaction appeared when it was exposed to immune serum after the sixth thawing.

The Effect of Phenol-Saline

It was found that 1/1000 merthiolate, either in the medium or mixed with the tissue, was insufficient to prevent bacterial growth on the plates. Phenol saline, 0·5 per cent., added to tissue suspensions or to pieces of tissues after being placed in the basins, prevented bacterial growth without interfering with the precipitin reaction.

Specific Diffusible Antigen in Tissues

The following experiments were carried out with myxoma tissue. Employing the hexagonal pattern, the central basin contained myxoma immune serum, and basins 1, 3 and 5 contained myxoma infected tissues. The material under test was placed in basins 2, 4 and 6.

When the material contained no specific antigen i.e., normal tissue, there were no precipitin line or lines between basins 2, 4 and 6 and the central basin. Precipitin line or lines produced by the control positive antigen in basins No. 1, 3 and 5 extended almost to the rim of basins 2, 4 and 6 forming a triangle (Figs. 2, and 3, top left). When the specimen was positive, i.e., oedematous myxoma tissue, the line or lines produced by all antigen/antibody reactions joined in a hexagonal form (Figs. 2 and 3, top right). When the specimen was weakly positive and not strong enough by itself to produce a reaction, the line or lines produced by the positive controls in basins 1, 3 and 5 were found to bend before reaching basins 2,

4 and 6. The bend was characteristic in its formation (Figs. 2 and 3, bottom left).

Fig. 2

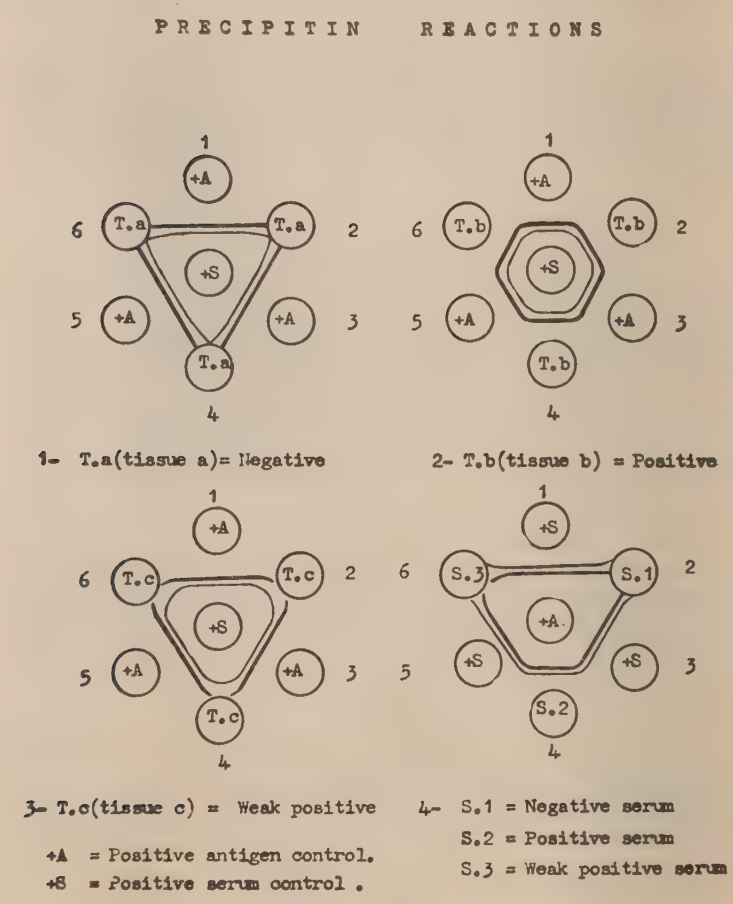


Diagram illustrating possible reactions in the precipitin test.

It has not been found possible to devise a method for the preparation of antigens applicable to all diseases. The tissue of choice varies from one disease to another and also depends upon the time after infection at which the tissues are harvested. A dilution of 1/5 or 1/10 of an HCC infected liver suspended in 0·5 per cent. phenol saline produced a definite reaction, but a similar or even a stronger dilution of 1/2 swine fever (SF) infected pancreas was too weak. With swine

fever it was usually necessary to place small pieces of tissue in the basins in order to produce a reaction.

In this investigation, a wide range of tissues were used from each virus-condition, viz, brain, spinal cord, spleen, lymph node, liver, gall bladder, peritoneal fluid, thymus, pancreas, urinary bladder, kidney, adrenal, lung, stomach, intestine and heart muscles, in an attempt to find out the most suitable tissue for each virus infection. While the liver and peritoneal fluid were suitable as a source of virus-antigen for the HCC reaction, they were not so with others. Mesenteric lymph node or stomach was suitable for DCX. Lymph nodes were suitable for DCX, HCC and LCM, but not for MYX or FIB, where the local skin lesion was ideal. The pancreas from swine fever infected pigs gave a more marked reaction than other tissues. No reaction has yet been demonstrated with Aujesky disease infected tissues or those of "virus pneumonia of pigs". Although some tissues e.g. spleen, liver and lung may contain precipitin antigen, the results were usually obscured by the haemolytic diffusion around the basin and in many cases it was not possible to read the result.

There were no reactions between normal sera and any of the virus antigens tested, nor between normal tissues and the viral antisera.

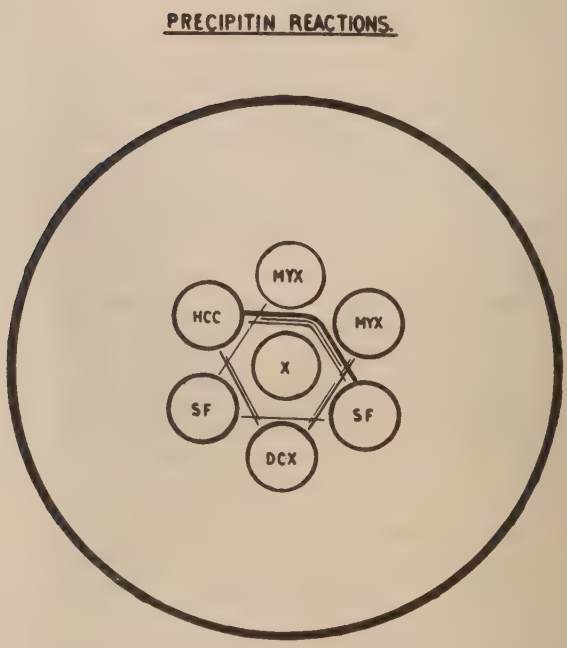
Specificity of Antigens Prepared from Different Virus Infections

In these experiments the central basin was filled with a mixture of myxoma, swine fever, canine distemper and Rubarth's disease specific immune sera. Basins 1 and 2 were filled with myxoma antigen, basins 3 and 5 with swine fever antigen, basin 4 with canine distemper antigen and basin 6 with Rubarth's disease antigen (Fig.4).

The first reaction to appear in four hours was the myxoma between basins 1 and 2 and the central depression. The lines produced by this reaction were found to curve and unite basins 1 and 2 which indicated identical antigenicity. The myxoma lines were found to extend almost to the rim of basins 3 and 6, both of which contained swine fever and Rubarth's disease antigen respectively. The lines produced by myxoma, swine fever, canine distemper and Rubarth's disease were found to be crossing each other, indicating no antigenic relationship between them.

The antigenic relationship between fibroma and myxoma can be demonstrated by placing a mixture of fibroma and myxoma antisera in the central basins of two units (Fig. 5). In plate 1, myxoma antigen was placed in basins 1, 3 and 5 and fibroma in basins, 2 4 and 6. In plate 2, myxoma antigen was placed in basins 1, 2 and 4 and fibroma in basins 3, 5 and 6. It will be seen that when two myxoma basins were next to each other the lines produced were bent as in unit 2. The same happened with two fibroma basins (plate 2). When a myxoma was placed next to a fibroma containing basin the lines produced by the myxoma appeared to combine to produce one line bending with the fibroma line, thus indicating antigenic relationship.

Fig. 4



Specificity of the precipitin reaction.

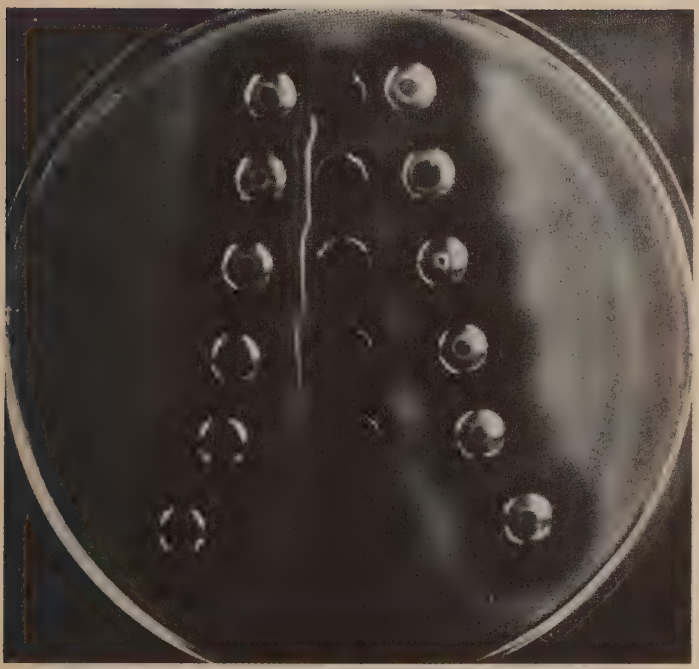
The Presence of Precipitin Antibodies

A known virus antigen, e.g., myxoma, was placed in the central basin, and known immune serum was placed in basins 1 3 and 5. Unknown sera placed in basins 2, 4 and 6. The results obtained were very similar to those obtained under the heading of testing antigens. Positive sera, e.g. myxoma, could be titrated in this test. Dilutions were made in tubes and then transferred into the respective basins. Titres were usually low (1/2 to 1/32). However, in most of the work the test was used as a qualitative one employing undiluted sera only.

CONCLUSIONS

The paper describes a modified technique of Ouchterlony's gel diffusion precipitin test. The results obtained by testing some virus-

Fig. 1



The effect of the distance between basins containing antigens and basins containing their specific antibodies on the appearance of the precipitin reaction. Left basins contained MYX immune serum: right basins contained normal rabbit serum: middle basins contained MYX virus-antigen. Photograph taken at 48 hours.

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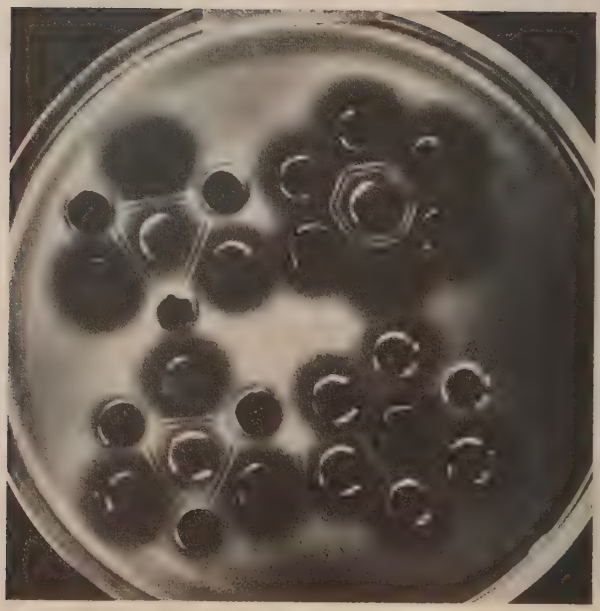
Fig. 3



Photograph taken 20 hours after reagents added to plate as in Fig. 2. In addition there are two additional reactions between basin 4, unit 2 (top right) and basin 1, unit 4 (bottom right) and between basin 3, unit 3 and basin 5, unit 4.

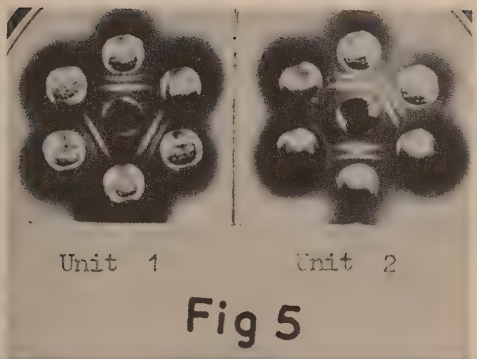


Fig 5

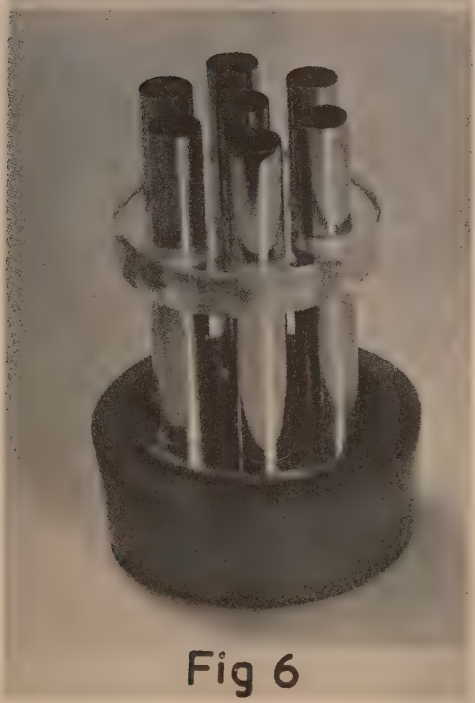


Fig 6

Fig. 5. Antigenic relationship between two immunologically related viruses (myxoma and fibroma of rabbits). Photograph taken after incubation overnight at room temperature.

Fig. 6. The cutting unit.

antigen antibody reactions are recorded. The viruses under study produced specific precipitin reactions, which varied in their nature, number of lines, time of appearance and sharpness of the reaction.

There were no cross reactions between any of the viruses studied with the exception of myxoma and fibroma viruses.

The simplicity of the test and the ease with which the results can be read suggest that this method will be of considerable value in studying antigen/antibody reaction in many virus diseases.

ACKNOWLEDGMENTS

The author wishes to express his thanks to Dr. A. W. Stableforth and Mr. F. D. Asplin for their interest, Mr. A. A. King for technical assistance, Mr. E. E. Clements for photographic assistance, and Mr. N. Burton for making the cutting appliance.

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CONTAGIOUS PUSTULAR DERMATITIS

III. Experimental Infection

By

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INTRODUCTION

The rabbit is widely used in the study of vaccinia and other poxes, and several workers have attempted to transmit contagious pustular dermatitis (C.P.D.) to it by inoculation of the scarified skin. The majority, however, failed to set up the disease (Zeller, 1920; Aynaud, 1923; Blanc, Melanidi and Caminopetros, 1923, 1924; Jacotot, 1926; Glover, 1928, 1944; Howarth, 1929; Boughton and Hardy, 1934). Lanfranchi (1925) claimed to have infected young rabbits on the lips. More recently Blanc and Martin (1933), Blaizot (1937), Borcila and Isopescu (1937), Bennett, Horgan and Haseeb (1944) and Horgan and Haseeb (1947) reported a more or less similar infection after inoculation on the scarified skin. All except the first and the last named authors omitted to confirm their findings by transmission back to sheep. Even in the two cases where it was tried, the material was taken from the first rabbit passage and the experiment was thus open to the objection that the virus in the original inoculum could easily have survived on rabbit-skin. However, as will be shown later in this article, all these workers had most probably succeeded in transmitting the disease to the rabbit. The egg has not been extensively used and the few reports in the literature contain conflicting information. Borcila and Isopescu (1937) observed numerous bullous vesicles filled with clear fluid on the chorio-allantois 48 hours after inoculation and considered these to have been caused by the virus. Beveridge and Burnet (1946), on the other hand, briefly mentioned that after inoculation in chick embryos by various methods, they failed to see any indication that the virus had multiplied.

In the present communication, serial passage of the virus on rabbit-skin, with back-transmission to sheep at appropriate stages, is reported along with a description of the naked-eye, histological and serological responses, together with the results of infection experiments in the fertile egg.

METHODS

The two strains of the virus used for this work have already been described (Abdussalam and Cosslett, 1957). One of them (U.F.) is the same as employed by Glover (1928), Bennett *et al.* (1944) and Horgan and Haseeb (1947) in their experiments with rabbits.

Rabbits. Horgan and Haseeb (1947) considered the breed of rabbits to be of great importance for experimental infection with C.P.D. even though they employed a single breed throughout their experiments. In the present work this factor was not found to be of much consequence so

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long as the rabbit was in good condition and had a large unpigmented area of skin for inoculation. A large majority of animals used was a mixture of the Belgian Hare, Old English, Blue Beveren and the Copenhagen breeds. A few pure Copenhagens and Dutch × Himalayan crosses were also used. No differences in lesions attributable to breed differences were noticed.

For inoculation on the skin, an area on the flanks and back was divested of hair. For this purpose shaving, depilatory chemicals and pulling out of fur was tried and the latter was found to be the least objectionable of the three methods. In most cases, the injury to skin ascribable to pulling out of fur was negligible. It consisted of a rapidly developing erythema, and slight, diffuse thickness, both of which completely subsided within 24 hours. Shaving and chemical depilations were both followed by severer reactions.

For inoculation by scarification, the skin was lightly abraded with the freshly cut and flamed edge of a wire-gauze held in an artery forceps. The inoculum was usually dropped on the area with a pipette and uniformly spread with the wire-gauze or a glass-rod. Scarification of the skin with a needle was tried and abandoned as the results were irregular, but it was used for inoculation on the oral mucous membrane. For intradermal inoculation, the technique commonly used in Schick test was employed, 0·1 to 0·2 c.c. being inserted at each inoculation. In corneal inoculation, performed under general anaesthesia, the cornea was scarified with a sharp needle and the inoculum dropped on to it. Usually only one cornea was inoculated, the other being left as a control. In due course, the rabbit was sacrificed, the eye-ball dissected out and the cornea removed for histological examination. In some cases, the entire eyeball was macerated in a glass jar in the presence of formalin vapour for 24 hours. If the corneal surface was touched with a clean slide, as in making impression smears, a sheet of corneal cells was removed which was fixed in methyl alcohol or osmic vapour and stained by Giemsa's method. Intra-testicular inoculation was also performed on one side only.

The inoculum in initial infections consisted of varying dilutions of e.b. suspensions comparable in opacity with Brown's No. 4-6, in McIlvaine's buffer solution (M 250, pH 7·0 to 7·2). For serial passaging, pieces of infected rabbit skin were ground in roughly 30 to 50 times their weight of buffer solution, centrifuged at low speed for 5 minutes and the supernatant used as inoculum. For collection of the infected skin, rabbits bearing lesions of suitable age were sacrificed and the infected area bearing confluent lesion excised; discrete lesions were clipped off with curved scissors. Infected skin was generally preserved in buffered glycerine (autoclaved mixture of equal parts of glycerine and McIlvaine's buffer, pH 7·0) at 0 to 4°C. and found to be infective up to 125 days (maximum period tested). In the earlier part of this work, suspensions of infected skin were always etherised according to the method described by Bennett *et al.* (1944) to keep down bacterial contamination, but later it was found to be unnecessary. As the rabbit lesions do not become pustular, pyogenic organisms do not seem to cause marked contamination. Fresh or preserved rabbit lesions set up a similar infection with or without etherisation. Material from sheep, particularly after pustulation had set in, however, always needed etherisation.

All the usual precautions regarding isolation of infected rabbits were taken and particular care was exercised to keep out vaccinia. When that

virus was handled other experiments were temporarily suspended, and its inoculation in rabbits was avoided so far as possible. No discrepancy ascribable to contamination with vaccinia was noticed.

Eggs. In general the methods described by Burnet (1938) and Beveridge and Burnet (1946) were followed. In the first series the standard inoculum was 0·05 c.c of a 1/500 or 1/1,000 dilution of the stock elementary body (e.b.) suspension from the U.F. strain equivalent to Brown's tube 5 in McIlvaine's buffer solution (pH 7·0). For serial passage, two or three infected membranes were ground in approximately 50 volumes of the buffer solution with or without sterile sand and centrifuged for 3 to 5 minutes at 2,000 r.p.m.; 0·05 c.c of the supernatant was inoculated in each egg. Temperatures of 35°, 36° and 37·5°C. were tried but no difference in the number or type of lesion was found; the chorio-allantois, however, seemed to be more oedematous at the lower temperature. The incubation was, therefore, fixed at 36°C. Unless otherwise stated the membranes were harvested at the end of 3 days. For sectioning and permanent mounting the technique described by Burnet (1938) was followed.

In the second series of experiments, a 1/50 dilution of a stock e.b. suspension of the W strain to which crystalline penicillin estimated at 25 to 50 units per egg was added as a bacteriostatic agent to the initial inoculum, was used. The eggs were incubated at 37°C.

Inoculation on the chorio-allantois was the method employed throughout, except for one experiment where inoculation was made into the amniotic sac. In the latter, no naked-eye effects were noticed.

RESULTS

Experimental Infection in Rabbits

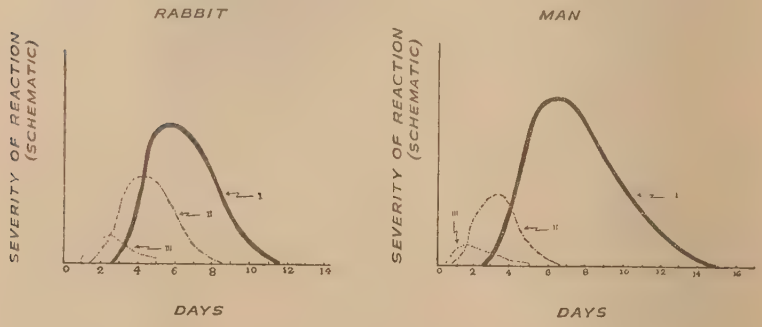
Scarification

No difficulty was experienced in infecting rabbits with a 1 : 50 dilution of washed e.b. suspension, fresh or preserved rabbit-skin lesions or etherised fresh scrapings of 4 to 6-day sheep lesions (U.F. Strain) and the infection was serially passaged up to the 22nd rabbit passage. In the first 18 passages, fresh material collected on the fourth or fifth days after inoculation was inoculated on to the next rabbit: thereafter, it was passaged after varying periods of storage in buffered glycerine in the cold room. Material from the fifth, ninth and the eighteenth passages set up the typical disease in lambs. In each of the last two tests, a lamb recovered from C.P.D. was included and failed to show any specific reaction. No change of virulence for sheep or rabbits was noticed as a result of this passage.

Macroscopic appearance. The following description of the naked-eye skin response is based on observations on 28 rabbits which were allowed to recover and several others sacrificed at varying intervals, mostly on the fourth or fifth day, after inoculation. In many cases, the experiments were controlled by scarifying another area on the skin and leaving it un-inoculated or smeared with buffer solution or sterile broth. The infected area showed moderately marked erythema and papulation on the third day after inoculation. In a single case, erythema was noticed 48 hours after inoculation but papules appeared only on the third day;

rarely, erythema and papulation were first noticed on the fourth day. The papules were only slightly raised over the skin surface and were liable to be missed if the skin was pigmented or injured by vigorous scarification. They were confluent or discrete according to the virus content of the inoculum, but even in heavily infected areas, a few discrete ones could be seen on the borders. On the fourth day the papulation became more marked and showed a well defined margin of congestion on the border of the lesion. Sometimes on the fourth but generally on the fifth day the papules became covered with a dull, yellowish-white scaly scab which increased in thickness the following day. At this stage the central parts of the more confluent lesions produced a serous or blood-tinged exudate, but the discrete lesions generally remained dry. No frank vesicular stage was observed. After the sixth day the reaction rapidly subsided and the scabs fell off in small scales or minute lumps leaving the skin clear but slightly thickened by the ninth to the twelfth day after inoculation. The course of the reaction is diagramatically represented in Fig. 1. In a majority of rabbits the peak of the response, as judged by the naked-eye reaction, was reached on the fifth day. No change in this syndrome was noticed during 22 serial passages in the rabbit, or when the second strain of the virus ('W') was used.

Fig. 1.



Diagramatic comparison of three types of reaction shown by the rabbit and man in C.P.D. I, normal; II, accelerated; III, 'vaccinoid' reaction.

None of the control areas developed lesions indicating that the disease remained localised and there were no secondary lesions in the oral cavity as is the case in sheep (Glover, 1928).

Histology of the skin response. The earliest changes, observed two days after inoculation, consisted of a thickening of the epidermis caused mainly by intercellular oedema in the deeper, and swelling of cells in the superficial, layers. There was no proliferation at this stage. The corium showed infiltration with a few mono-nuclear cells.

On the third day, the epidermis was noticeably thickened both as a result of proliferation and swelling of the cells. The superficial cells of the Malpighian layer showed reticular degeneration and their nuclei were shrunken, collapsed and distorted or fragmented. A part of this change may be the normal process of keratinization going on in these cells. The corium showed congestion, even haemorrhages, and infiltration with polymorphs, eosinophils and mono-nuclear inflammatory cells.

On the fourth and fifth days the reticular degeneration of cells of the Malpighian layer extended deeper but rarely reached the basal layer. In the meantime, the superficial cells became necrotic and formed an eosinophilic mass within the stratum corneum and accompanied by exudates containing some inflammatory cells. The number of cells infiltrating the dermis, particularly the mono-nuclears, increased but congestion subsided (Figs. 2 and 3). There was no break-down of cell membranes to form large vesicles, nor any pus formation at any stage. This would explain the apparent transition of the papule stage into scab formation described as a naked-eye response.

Histological evidence of healing started by about the sixth day after inoculation. The necrosed epidermis, mixed with exudate and remains of infiltrating cells, became sharply demarcated from living cells. The corium showed proliferation of connective tissue-cells, but the inflammatory cells were fewer than before. In the next few days, the basal layer of epidermis produced more cells and the overlying scab fell off leaving a thickened skin covered with epithelium. As the basal layer remained intact there was no pock-mark or scar formation, thickening of the skin being due to proliferation of the connective tissue in the corium.

Although the type of degeneration of the epidermal cells was the same (reticular) as in sheep, yet no cytoplasmic or nuclear inclusions were seen. There was no balloon degeneration of these cells.

Intradermal Route

Most recent workers on immunity in pox diseases, particularly vaccinia, have preferred to use intradermal injections for the titration of the virus as this method gives more constant results. This mode of inoculation was, therefore, given particular consideration in the present investigation.

On spots inoculated with 100 to 1,000 m.i.d. of the virus, the button-like swelling, caused by the injection, disappeared after a slight initial enlargement, in about six hours. A pink erythematous spot, 5 to 10 mm. in diameter, appeared at the site on the third day after inoculation. It soon changed into a flat circular papule which increased in thickness and diameter till about the sixth day when its maximum severity was reached. In the next one or two days a yellowish-white scaly covering appeared on its surface which increased in thickness and was cast off by the tenth day. This left a pale-pink thickening which disappeared by about the fifteenth day. The lesion remained strictly localised and no generalisation to other parts of the skin was observed. The lesion thus set up by intradermal inoculation had a more prolonged course than that following scarification. On control spots inoculated with sterile buffer solution, the initial swelling disappeared in a few hours and thereafter the skin remained normal in appearance.

This method of inoculation was chiefly employed for titration and virus-neutralisation tests. Prolonged serial transmission was not carried out because extraneous infection was more prevalent with this method than with scarification. It was attempted in one case, but abandoned at the third passage when the centre of the

lesion developed a pustule 24 hours after inoculation. Material from the second passage produced typical lesions on a susceptible lamb, but gave negative results on a lamb convalescing from C.P.D.

The response to graded doses was more marked when the virus was inoculated intradermally than by scarification. In the latter case, concentrated doses tended to produce a confluence of the lesions, but failed to affect either the incubation period or the course. After intradermal inoculation, however, the incubation period, the course of the disease and even the character of the lesions varied according to the virus content of the inoculum. Although responses of individual rabbits varied in this respect, the general tendency was a prolongation of the incubation period and a shortening of the course of the lesions with a decrease in the virus content. The typical response of a rabbit to graded doses inoculated with each of the virus dilutions at two spots on the skin is shown in Table 1. The change in the naked-eye character of the lesion was also a noticeable feature. A concentrated dose of the virus caused necrosis of a considerable central part of the papule as evidenced by a darker colour and the subsequent formation of a depressed area covered over with an adherent dark scab which delayed the healing for several days. Smaller doses tended to reduce the scaly scab formation, to the extent that very small quantities of the virus produced a transient papule only.

TABLE 1

RESPONSE OF RABBIT TO INTRADERMAL INOCULATION OF GRADED DOSES OF C.P.D. VIRUS

Dilution of stock e.b. suspension	*Period of incubation (days)*	*Course* of the lesion (days)*	*Remarks*
1 : 10^{-1}	2	20	Central part necrosed and depressed.
1 : 10^{-2}	3	19	
1 : 10^{-3}	3	15	
1 : 10^{-4}	5	12	
1 : 10^{-5}	6	11	
1 : 10^{-6}	7	11	No scabs or scaby covering.
1 : 10^{-7}	—	No lesion	
Sterile buffer soln. only	—	No lesion	

*Calculated from the day of inoculation.

Corneal Route

During serial passage of the virus in rabbits by skin scarification, several animals were also inoculated by scarification on one cornea

with a portion of the same inoculum; the other cornea was usually scarified but left uninoculated. The inoculated eyes developed no naked-eye lesion, but on the third or fourth day the superficial layers of corneal tissue were easily detached with a piece of cotton-wool wetted with saline, leaving a large shallow ulcer. Impression smears of the cornea stained with Giemsa showed no abnormality except granular material of a reddish-violet colour in the cytoplasm of a few of the epithelial cells. Sections of infected cornea, stained with haematoxylin and eosin, showed proliferation of the epithelium in the inoculated areas. The normal 4 to 5 layers increased to ten layers of cells in some parts (Fig. 4). There were no cytoplasmic inclusions as in vaccinia, nor was there any involvement of the deeper tissues of the cornea or any infiltration with inflammatory cells. An unsuccessful effort was made to demonstrate e.bs. in impression smears after staining by Paschen's method.

As a check on the methods used in the foregoing enquiry a rabbit, inoculated by analogous methods with vaccinia, developed the typical changes described by several workers (van Rooyen and Rhodes, 1940) and Guarnieri bodies in large numbers could be seen both in impression smears and in sections. Impression smears also showed numerous e.bs. when stained with victoria blue.

Infection by Other Routes

Inoculation into the testicle and by intranasal instillation respectively of heavy doses of the virus, in the form of e.b. suspensions, did not produce observable pathological changes. Several small areas on the inside of lips, on the gums and on the tongue of a rabbit were also scarified and inoculated with a suspension of infected rabbit skin. Another portion of the same inoculum was inoculated on the skin of the same rabbit. Only the skin developed typical C.P.D. and the areas on the oral mucous membrane remained healthy during the 10 days of observation.

Titration of the Virus

Preliminary titrations by scarification indicated that the m.i.d. for rabbits was 200 to 2,000 times that required for sheep. In view of the variations in the results this method was abandoned. The rabbit was suitable, however, for titration experiments if the intradermal route of infection was employed.

Immunity in the Rabbit

In sheep an experimental or natural attack of C.P.D. leaves a strong to complete immunity to re-infection. This led certain workers to suppose that such was the case in the rabbit (Horgan and Haseeb, 1947). Nevertheless, Blanc and Martin (1934) had concluded from a single experiment that there was no immunity in the rabbit except at the site of inoculation and the course of the disease in four successive infections in the same rabbit did not differ from the primary

attack. Blaizot (1937) was also able to re-infect a rabbit immediately after recovery from a primary infection. His protocols show that the course of the second infection was more rapid than the first, though he did not comment on this fact. In the present investigation it was found that a rabbit immediately on recovery from infection on a sizeable area of skin showed only partial immunity to re-infection as evidenced by "accelerated" reactions. This immunity, however, disappeared in about seven weeks' time.

At various times in the course of the present work, rabbits which had recovered from typical C.P.D. affecting a large area of the skin became available for re-test. They were inoculated by scarification with about 100 to 1,000 m.i.d. along with normal rabbits. Two animals were subjected to a third infection and one to a fourth. The type of reaction is shown by the incubation period and course of the disease, the latter being the time taken for the majority of the scabs to fall off. The U.F. strain was used for the primary as well as the subsequent infections. Table 2 shows that the primary infection in all the rabbits appeared regularly after an incubation period of three days and healed in 9 to 11 days' time, the peak being reached on the fifth day. If they were re-infected within four weeks they showed an "accelerated" reaction which appeared after an incubation period of 2 days and healed in 7 to 8 days, the peak being reached on the fourth day. A higher degree of immunity was shown by other rabbits in which the reaction was even more rapid. The tests also showed that if a sufficient time (30 to 186 days), was allowed to elapse, the partial immunity disappeared and the rabbit showed a typical reaction comparable with that of a healthy rabbit. No evidence of natural immunity was observed in any of the rabbits employed. Various areas of the skin were also tested including the sites of previous infection and the lesions which developed were indistinguishable. This is not in accordance with the findings of Blanc and Martin (1934) who observed some local immunity.

Infection Experiments with other Animals

Unsuccessful infection of the guinea-pig and mouse with C.P.D. has been reported by Blanc *et al.* (1923, 1924), Aynaud (1923), Jacotot (1926), Glover (1928), Blanc and Martin (1933) and Blaizot (1937). In the present investigation entirely negative results were obtained even though massive doses of the virus in the form of e.b. suspensions were used.

Guinea-pigs inoculated on the skin by scarification and intradermally on the foot-pad developed ringworm due to *Trichophyton gypseum* on the skin. Rabbit-passaged virus in the 13th generation failed to infect the guinea-pig. Blind passage up to three generations also failed to elicit any specific reaction.

Mice inoculated on the skin or intranasally did not react. Impression smears of inoculated lungs stained by Paschen's method failed to show any elementary bodies.

TABLE 2

IMMUNITY TESTS IN RABBITS

All times in days

Rabbit No.	*First infection*		*Interval between 1st & 2nd infections*	*Second infection*		*Interval between 2nd & 3rd infections*	*Third infection*		*Interval between 3rd & 4th infections*	*Fourth infection*	
	incubation period	*Course*		*incubation period*	*Course*		*incubation period*	*Course*		*incubation period*	*Course*
A.76	3	10	11	2	7[1]	—	—	—	—	—	—
B.53	3	10	14	1	4	—	—	—	—	—	—
B.43	3	10	20	2	8[1]	186	3	10	—	—	—
B.62	3	10	28	2	7[1]	10	1	5[1]	170	3	11[1]
B.53	3	11	50	3	10	—	—	—	—	—	—
A.98	3	9	80	3	10	—	—	—	—	—	—
A.97	3	11	120	3	10[1]	—	—	—	—	—	—

Viability of the challenge inoculum, in each case, was controlled by including healthy rabbits in the test.
Periods of time (course, intervals, etc.) in all cases were calculated from the day of inoculation onward.
[1] Scabs produced typical C.P.D. on lambs.

An adult hedge-hog (*Erinaceus europaeus*) inoculated intradermally on the tongue and foot-pads, as well as by scarification, showed no reaction up to four days when it died of unknown causes. Microscopic examination of sections of its tongue failed to show any abnormality.

Effect of Trichophyton Infection

Recently, Selbie (1944, 1945, 1946a) reported transmission of two strains of C.P.D. to the rabbit. One he passaged serially in 26 animals, but failed to "re-transmit" the other. Scarification was the only successful mode of inoculation, and the disease developed after a variable incubation period of 6 to 20 days. Although the original material was readily transmissible to sheep and goats and set up typical C.P.D., the rabbit passaged material was entirely non-pathogenic for ovines. After a preliminary failure, Selbie (1945) was able to transmit the rabbit disease to the guinea-pig. The course of the disease was, on the whole, shorter than in the rabbit.

An examination of his protocols indicates that Selbie's disease is very different from experimental C.P.D. in rabbits described in the present article and that reported by earlier workers. It has a much longer and variable incubation period, is considerably more severe and runs a much longer course. Selbie (1945) attributed these differences to the strain of rabbits used, but this seems hardly plausible, especially as it leaves the inability to transmit the disease back to sheep unexplained. In the course of the present investigation, a ringworm fungus (*Trichophyton gypseum*) was isolated from Selbie's material and a disease indistinguishable from that described by him was set up with cultures of the fungus from this source and from cattle. *T.gypseum* was readily demonstrable in the lesions on rabbits and guinea-pigs at all stages. A natural resuscitation of ringworm in experimental guinea-pigs was observed and certain other observations and experiments bearing on the subject were made, which leave little doubt that Selbie was dealing with ringworm rather than C.P.D. in his rabbits and guinea-pigs.

Transmission to Eggs

Infection of Chorio-allantois with C.P.D. Virus

It was noticed that under identical conditions, 12-day were more suitable than 9 or 10-day embryos since in the former a higher proportion became infected and the lesions were more marked.

Naked-eye appearances. The lesion was essentially a mild one and even under optimum conditions it was not possible to infect all the eggs inoculated. A maximum of about three-fourths showed observable response when 12-day embryos were inoculated with e.b. suspensions and subsequently incubated at 36°C. At 24 hours there was usually no macroscopic response except slight oedema. At 48 hours this oedema was increased and the membrane showed numerous pin-point opacities, more numerous peripherally, of a maximum

diameter of 0·25 m.m. Even when confluent, individual points could be distinguished, possibly because of their small size. At 72 hours the oedematous membrane had become somewhat hazy and the individual points thereby rendered less conspicuous. Some showed a non-specific ulcer in the centre of the "dropped" membrane. At 96 hours the number of individual lesions was considerably smaller and the membrane had become thinner though the haziness still persisted. The most characteristic changes were, therefore, seen at 48 and 72 hours after inoculation. There was no evidence of generalisation of the lesions and no specific mortality of embryos.

Histology. At 24 hours the ectoderm showed proliferation of ectodermal cells. At places, there were vacuoles of varying sizes in the cytoplasm of the outermost cells, but no inclusions were seen. There was no marked change in the underlying mesoderm except congestion and leucocytosis. At 48 hours, the ectodermal proliferation was somewhat more marked, but cells, at some places presumably centres of individual lesions, showed marked vacuolation of the cytoplasm. In many places these vacuolated cells were necrosed and subsequently shed, leaving crater-like depressions. The mesoderm now showed proliferation of connective-tissue cells and infiltration with leucocytes, as well as oedema. The endoderm showed proliferation in the neighbourhood of the more heavily affected areas of the ectoderm (Fig. 5). At 72 hours the shedding of ectodermal cells was even more marked but, due to continued proliferation, they remained 3 to 4 layers deep in the affected areas. The mesoderm at this stage showed more proliferation and formation of granulation tissue. The endoderm had also proliferated and was 3 layers deep in certain places with many of the remaining ectodermal and the connective tissue cells in the mesoderm showing brightly eosin-coloured round or oval bodies of varying sizes in their cytoplasm. There were no remains of erythrocytic nuclei in the neighbourhood of these "inclusions" so that they may be specific to C.P.D. virus (Figs. 6 and 7). They did not resemble the non-specific acidophilic inclusions mentioned by Goldsworthy and Moppett (1935) and commented on by Burnet and Lush (1936). At 96 hours the inflammatory changes had subsided and the proliferated endoderm was cast off. Smears of 2-day and 3-day lesions stained by Paschen's method showed numerous regular particles which might have been e.bs., but they could not be definitely identified.

Serial Transmission of C.P.D. in the Egg

In the first series, the infection was passaged serially in 12-day eggs three times. As the egg-passage advanced the number of eggs showing infection and the severity and density of the lesions decreased. In two series the visible response disappeared after the fourth and in one after the third passage. In the first of these series, "blind" passage was continued up to the ninth generation without revival of the infection. Membranes of the fourth generation were infective for

a lamb, but the sixth and ninth generations were non-infective. Infectivity disappeared, therefore, at about the same time as the naked-eye response.

In the second series of experiments, a heavier initial dose using stock e.b. suspension instead of a 1 to 500 or 1 to 1,000 dilution was used. No observable response was seen beyond the fourth generation.

DISCUSSION

Zeller (1920), Aynaud (1923), Blanc *et al.* (1923, 1924), Jacotot (1926), Glover (1928, 1944), Howarth (1929), Boughton and Hardy (1934) failed to infect the skin of rabbits with C.P.D. Details of the preparation of infective material and the method of inoculation used by these workers are not available in most cases, but a clue is sometimes available in other work reported along with these experiments. Glover (1928), for example, in his sheep experiments used scabs collected 18 days after infection and dried over sulphuric acid for 3 days, and presumably he used a similar source of the virus in his rabbit experiments. It is well known that scabs collected so late in the course of the disease are poor in virus content (Bennett *et al.*, 1944), and deteriorate further during desiccation and storage. Some failures to infect rabbits may, therefore, have been due to the use of material with a low virus content. Since the reaction is relatively mild the possibility that it may have been overlooked cannot be excluded; furthermore, pyogenic organisms in the inoculum may have interfered with the establishment of the virus in some of the investigations reported.

Lanfranchi (1925) claimed successful infection of the rabbit, but it is doubtful whether he actually set up the disease. He did not test the infectivity of the lesions for sheep, nor did he study the histological changes, and his lesions, so far as known, differ from those subsequently reported by other workers. More recently Ishii *et al.* (1953) have reported successful transmission of the disease to the rabbit by lip inoculation, but the details of their work are not readily available. Contrary to the earlier experience of Blanc *et al.* (1923), Blanc and Martin (1933) reported successful transmission of a Moroccan strain of C.P.D. to a rabbit. A somewhat similar infection was subsequently reported by Blaizot (1937), Borcila and Isopescu (1937) and Bennett, Horgan and Haseeb (1944). All these workers used fresh or freshly dried material from early lesions of sheep for inoculation. The only attempts to prove the specificity of the lesion by successful re-transmission of the disease to sheep were made by Blanc and Martin (1933) and Horgan and Haseeb (1947). As the material for re-transmission was taken from the first rabbit inoculated with sheep virus, the experiments are open to the possible objection that the virus in the original inoculum could have survived on the rabbit's skin. Nevertheless it seems highly probable that they had established the disease.

In the present investigation the infection was serially passaged in 22 animals, re-transmitted to susceptible sheep from the 18th passage and the virus demonstrated microscopically. Further evidence of the identity of the rabbit and sheep diseases was provided by a study of the histological and serological responses.

When the disease is induced in the rabbit by scarification, some of the interesting features are the regular incubation period of 3 to 4 days, which is the same as in sheep, the absence of frank vesiculation and suppuration, the mildness of the lesion and healing within 2 weeks after infection. A much higher dose of the virus is required to infect rabbits than sheep, thus making it less suitable for use as a test animal for the detection of virus. Infection of rabbits by intradermal inoculation, which has not been previously reported, is of particular value in the titration of virus and in serum neutralisation tests. The influence of graded doses of the virus is much more manifest in infection by the intradermal route than in infection by scarification and is somewhat similar to that observed in vaccinia (Ledingham, 1924). Of the other routes tried corneal inoculation was followed by a response possibly ascribable to the C.P.D. virus. In the absence of naked-eye changes this response is not of much practical interest.

In marked contrast to sheep, in which immunity to re-infection soon after recovery is strong to complete, the rabbit shows only a partial resistance, which, however, is temporary and disappears in 50 days' time.

C.P.D. virus produces only a mild response on the chorio-allantois and this decreases on serial transmission. It is so poor that the fertile egg is not of much practical importance in the study of C.P.D. unless it is subsequently found that it can be intensified. Of the previous workers, Glover (1946) had results similar to the foregoing, except that in his experiments the lesions disappeared after the second generation. The findings of large vesicular lesions has been reported (Borcila and Isopescu, 1937). There is little doubt that these were the non-specific "cystic swellings filled with clear fluid" of Beveridge and Burnet (1946) and may have been the effect of variation in the temperature of incubation. The infectivity tests of Borcila and Isopescu (1937), however, yielded results similar to those now reported. The first, second and third egg passages were fully infective for lambs, while the seventh passage produced only a doubtful reaction. It seems, therefore, that these workers had infected the egg in much the same way, but failed to recognise the minute lesion and mistook the non-specific, but more prominent, cystic swelling for it. Since this work was done, Lyell and Miles (1950) have reported lesions similar to those described in the present article, but Hart, Hayston and Keast (1949) and Macdonald (1951) failed to produce noticeable lesions. These apparently contradictory reports are to be expected in such a mild infection in which the virus titre of the inoculum or the presence or absence of some factor in the egg would materially affect the observable response.

CONCLUSIONS

The rabbit can be infected with contagious pustular dermatitis if a massive dose of the virus is applied on the scarified skin or inoculated intradermally. The infection can be serially transmitted.

Because of its lower susceptibility, the rabbit is less suitable than the sheep as a test animal when the presence of the virus has to be detected, but is suitable for virus neutralisation tests when the intradermal route of infection is employed.

Recovery from the experimental disease is followed by a partial resistance to reinfection which disappears in about 50 days.

The guinea-pig and the mouse are not susceptible to the virus of C.P.D. when inoculated by routes used in the rabbit.

It has been shown that in rabbits and guinea-pigs a skin reaction ascribed to the virus of C.P.D. was due to a ringworm fungus (*Trichophyton gypseum*) which occurred as a contaminant in experimental infection.

A mild lesion is produced on the chorio-allantois of 9 to 12-day chick embryos. The macroscopic and microscopic changes are described. The lesion regresses continually during serial passage in 12-day eggs and disappears by about the fourth generation.

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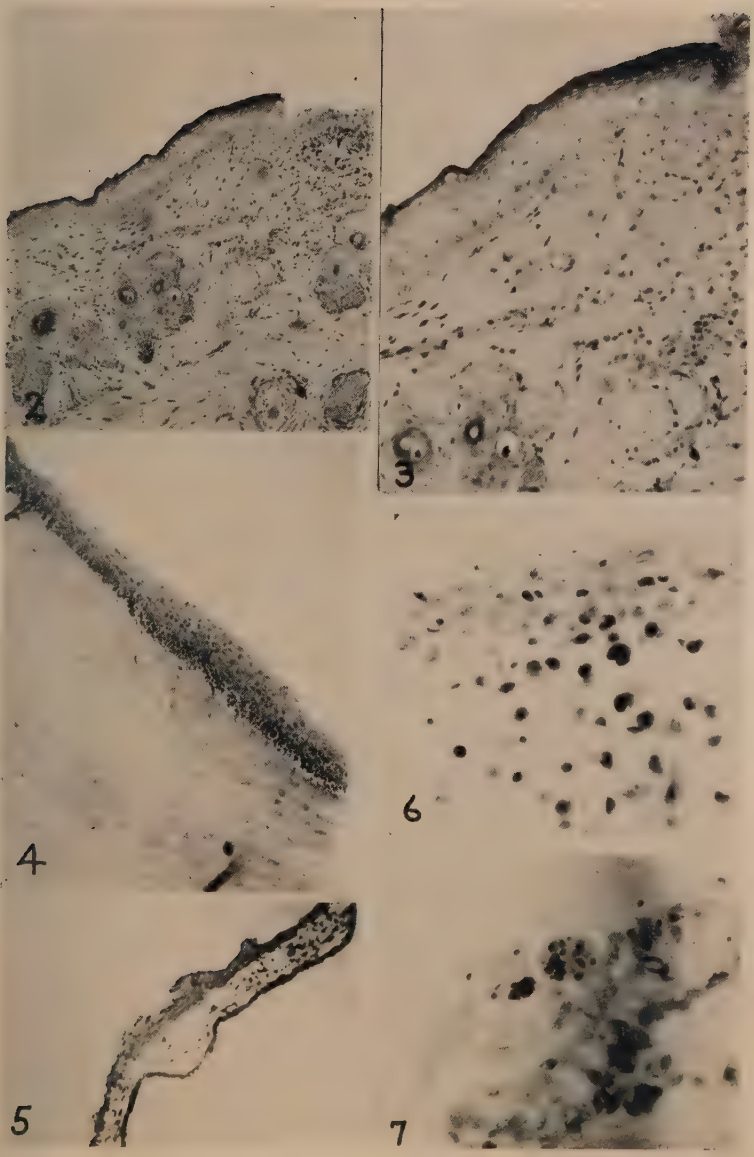

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LEGENDS TO ILLUSTRATIONS

Fig. 2. Four-day lesion on rabbit skin. Proliferation and reticular degeneration of epidermis and infiltration of corium by inflammatory cells. Part of the section was removed during section cutting. H. and E. x 90.

Fig. 3. Part of the section in Fig. 2, more highly enlarged. x 175.

Fig. 4. Part of rabbit cornea, 3 days after inoculation with C.P.D. virus. Proliferation of epithelium. H. and E. x 175.

Fig. 5. Section of 2-day lesion of C.P.D. on chorio-allantois. 'Crater' formation. H. and E. x 90.

Fig. 6. Eosinophilic inclusions in the cytoplasm of ecto- and meso-dermal cells in a 3-day C.P.D. lesion on chorio-allantois. H. and E. x 470.

Fig. 7. Eosinophilic inclusions in the cytoplasm of endodermal cells in a 4-day C.P.D. lesion on chorio-allantois. H. and E. x 470.

A HAEMOPHILUS-LIKE ORGANISM ISOLATED FROM PIG LUNG AND THE ASSOCIATED PNEUMONIC LESIONS

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INTRODUCTION

During the examination of pig lungs showing pneumonia, a case was encountered from which swine fever virus and a *Haemophilus*-like organism were isolated. Experimental inoculations with the *Haemophilus* showed that by itself it was apparently non-pathogenic, but that when combined with swine fever virus extensive pneumonia with a characteristic histological picture was produced.

This paper describes the *Haemophilus*-like organism and the pneumonic lesions with which it was associated.

MATERIALS AND METHODS

Bacteriological. The *Haemophilus* was originally isolated in association with a *Staphylococcus* on Bacto-tryptose ox-blood agar incubated aerobically at 37°C (Fig. 5). Further identification necessitated the use of other media and these are detailed below where a description of the organism is given. A bacteriological examination was made of all lungs, and special precautions were taken to provide conditions favourable for growth of the *Haemophilus*.

Identification of swine fever virus. Seitz EK filtrates of pneumonic lung were infective for pigs when inoculated intratracheally, intravenously, or intraperitoneally, and the disease produced in a total of 35 experimental pigs was typical of swine fever both clinically and at post-mortem examination. The virus was maintained through seven passages by inoculation of Seitz EK filtrates. Final identification of the virus was based on the fact that its pathogenic action was blocked by crystal violet vaccine, lapinised virus vaccine,† and by swine fever hyperimmune serum.

Histological. Tissues, fixed in 10 per cent formol-saline within three hours of slaughter or within twelve hours of death, were embedded in paraffin and stained with haematoxylin and eosin and by Gram's method. Eleven blocks, taken from the apical, cardiac, intermediate and diaphragmatic lobes, were examined from all lungs showing macroscopic evidence of pneumonia, and not less than four blocks were examined from all lungs in which there was no macroscopic evidence of disease.

Experimental animals. Recently weaned pigs from the Compton farm were used for experimental inoculation; they were housed in loose-boxes in the Isolation Compound during the period of experiment. Inoculations were by the intratracheal route under intraperitoneal nembutal anaesthesia with the animal on its back and the head slightly higher than the tail.

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†Rovac (Lederle).

Preparation of experimental inocula. Three pigs, (2, 3 and 4, Table 1 and Fig. 1) were inoculated intratracheally with 20 ml. of a 10 per cent suspension of unfiltered material from the original pneumonic lung (Pig. 1 in Table 1 and Fig. 1). The inoculum was made by grinding a weighed amount of pneumonic lung in a mortar with a little sand and adding M/15 phosphate buffer saline to make a 10 per cent suspension. The *Haemophilus* was isolated in pure culture from pig 4 in this series, and subcultures of this isolation were used for subsequent inoculations. Swine fever virus was also present in this lung and was used for infection of later pigs. The attempts to produce disease with the *Haemophilus* were made in two experiments, details of which are given in Table 1. The volume inoculated was in every case 20 ml. intratracheally.

In the first experiment the organism was grown aerobically at 37°C for 48 hours in 30 per cent pig serum broth. The reason for the selection of this medium was empirical because at this time little progress had been made with the identification of the organism. Although growth was slight in this medium it was judged to be sufficient for pig inoculation. Twenty ml. of this culture were inoculated into three pigs, (5, 6 and 7). The inoculum containing swine fever virus consisted of 20 ml. of a 10 per cent suspension in serum broth centrifuged at 1,500 r.p.m. for 10 minutes and passed once through a Seitz EK filter; this was inoculated into three pigs, 24, 25 and 26. The inoculum containing both the *Haemophilus* and the swine fever virus consisted of 10 ml. of *Haemophilus* culture and 10 ml. of Seitz EK filtrate containing the swine fever virus, both prepared as above; this inoculum was used for pigs 15, 16 and 17. As controls in this first experiment pigs 12, 13 and 14 received 20 ml. of 30 per cent pig serum broth, and 22 and 23 were given 20 ml. of a Seitz EK filtrate of the *Haemophilus* culture.

In the second experiment the *Haemophilus* was grown aerobically at 37°C for 48 hours spread over the surface of 4 in. plates of the special medium (see below) prepared for encouraging growth of this organism. The growth from each plate was rubbed off with 2·0 ml. of M/15 phosphate buffer saline for the inoculation of one pig. Pigs 8, 9, 10 and 11 each received this inoculum. The inoculum containing swine fever virus was prepared by centrifuging at 1,500 r.p.m. for 10 minutes a 10 per cent suspension in M/15 phosphate buffer saline of pooled spleens from earlier pigs in the series and passing it once through a Seitz EK filter; the volume inoculated was 20 ml. and this was given to pigs 27, 28, 29 and 30. The *Haemophilus* was combined with swine fever virus by adding, for each pig, the growth from a 4 in. plate rubbed up in 2 ml. of M/15 phosphate buffer saline to 18 ml. of a Seitz EK filtrate of a 10 per cent splenic emulsion in phosphate buffer saline containing the virus; this inoculum was used for pigs 18, 19, 20 and 21.

RESULTS

Description of the Haemophilus-like Organism

The organism was a small pleomorphic Gram-negative rod, showing a predominance of coccobacillary forms after incubation for 24 hours. After 48 hours filamentous forms were sometimes observed (Fig. 4). Motility was not demonstrated, but capsulation appeared to be a feature. The organism was difficult to grow on primary culture and it was due to its powers of satellitism that it

was first noted. In association with a staphylococcus, colonies of up to 1 mm. were observed, but otherwise they were almost pin point in size. Examination under the colony microscope showed a raised periphery surrounding a depressed area with a prominent central papilla, particularly after incubation for 48 hours. The colonies were difficult to remove from the surface of the medium and appeared to embed themselves in it. Catalase was not produced. From its morphology and colonial appearance the organism appeared to be similar to *H. influenzae*, but its nutritional requirements were not the same. The following table summarises our findings when the *Haemophilus*-like organism and *H. influenzae* were cultured on various media:—

GROWTH REQUIREMENTS OF THE HAEMOPHILUS-LIKE ORGANISM ISOLATED FROM PIG LUNG AS COMPARED WITH H. INFLUENZAE

	Haemophilus-like organism	*H. influenzae*
Bacto-tryptose ox-blood agar	+ (72 hrs.)	—
Rabbit blood chocolate agar	+	+++
10% Boiled rabbit blood agar	—	—
1% Commercial* yeast extract agar	++	—
10% Boiled blood agar+1% commercial yeast extract	+++	—

*Oxo.

It is apparent from these results that the organism did not require the X factor found in blood pigments since it grew on the commercial yeast extract agar. Furthermore, it did not grow on a medium containing X factor alone, namely 10 per cent boiled rabbit blood agar. Its ability to grow weakly on blood and chocolate agars but not on boiled blood agar suggested that a thermolabile factor was required. However, this factor is not the typical V of *H. influenzae* since, although good growth was obtained in the presence of yeast extract plus X factor, this medium failed to support the growth of *H. influenzae*, presumably due to the commercial yeast extract lacking factor V. Mr. H. Proom of the Wellcome Research Laboratories, Beckenham, kindly examined the growth of this organism on a wide selection of media, including those used for *H. influenzae*. He was unable to obtain growth except on rabbit serum agar, heated blood agar +15 per cent rabbit serum, glucose serum agar and Löwenstein-Jensen medium, the last after incubation for 20 days. These findings confirmed our earlier ability to obtain some growth in 30 per cent pig serum broth. In summary, therefore it would seem that the nutritional requirements of this organism are difficult to define; a thermolabile factor present in blood, serum, and commercial yeast extract appears to be essential and this is also provided as a product of growth of the staphylococcus.

Lewis and Shope (1931) considered *H. influenzae suis* to be similar in its growth requirements to *H. influenzae* but Alexander

(1948) reported that a number of strains of *H. influenzae suis* examined by her did not require factor X. It is possible, therefore, that such strains may have been similar to the organism now being described. Rosher and Hardy (1953), using a special technique, studied giant colonies formed by various members of the haemophilus group. A culture of the haemophilus-like organism described above, was tested by them and they report that the giant colony formed bears no resemblance to numerous strains of *H. influenzae, H. influenzae suis,* or other members of the group they have studied by their technique.

For subsequent work and for the second experiment a special medium was used. In preparing this medium 10 per cent boiled rabbit blood in Lab Lemco broth was mixed with 4 per cent agar, and to this were added 10 per cent Oxoid bacteriological yeast extract in Lab Lemco broth to a final concentration of 1 per cent, and a Seitz EK filtrate of an 18-hour broth culture of a *Staphylococcus aureus* phage type 3A (Compton No. 204) to a final concentration of 10 per cent.

Experimental Inoculations with Pure Cultures of the Haemophilus-like Organism

In the two experiments, a total of seven pigs was inoculated intratracheally with pure cultures of the *Haemophilus* (Table 1 and Fig. 1).

No clinical abnormality was noted in any pig given either culture or control material. No macroscopic abnormality was found in any animals in these groups (Fig. 1). No pathological change was found in any lung from these animals, although a variable degree of non-specific increase in the cellularity of the alveolar walls, attributed to inoculation of foreign material, as described by Pattison (1956), was noted in some sections from animals that received all the different types of inocula. Neither the *Haemophilus* nor any other organism was cultured from these lungs.

Experimental Inoculations with Swine Fever Virus

It was observed in both experiments that inoculation of the *Haemophilus* alone caused no disease, but that when this organism was added to an inoculum containing swine fever virus a severe pneumonia was produced that had certain characteristics not associated with pure swine fever infection. In order to assess the pathological action of the *Haemophilus*, therefore, it was necessary first to determine the extent and kind of pneumonia that might be attributed to the swine fever virus alone. Alterations to this swine fever picture in animals inoculated with the *Haemophilus* plus swine fever virus might then be attributed to the action of the *Haemophilus*. In the two experiments a total of seven pigs was given swine fever virus intratracheally (Table 1 and Fig. 1).

Clinical observations. The disease produced was in every way characteristic of swine fever, with loss of appetite, lethargy, diarrhoea.

inco-ordination of the hind-limbs, skin lesions and cyanosis of the ears. Post mortem examinations were typical of swine fever.

TABLE 1

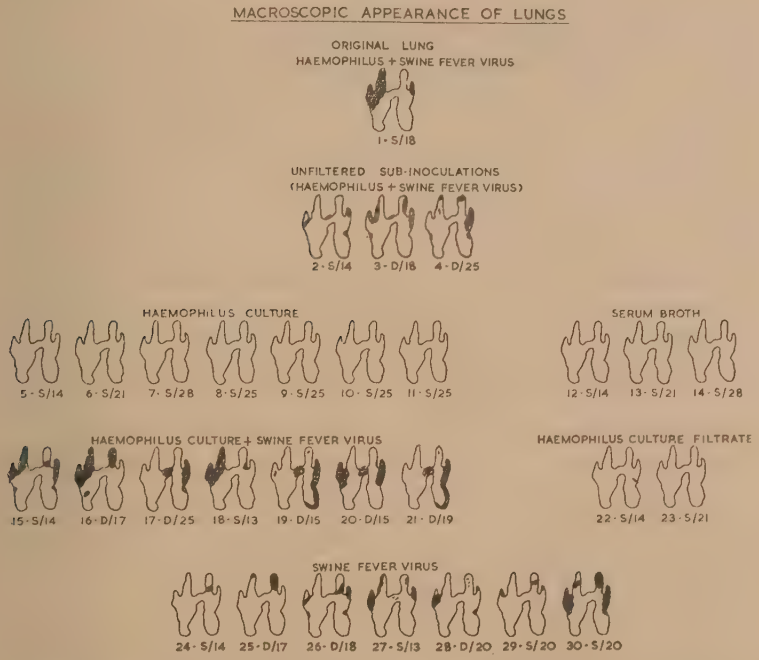
RESULTS OF INTRATRACHEAL INOCULATION OF PIGS

(a) Cultures of the *Haemophilus*-like Organism. (b) Swine Fever Virus. (c) The *Haemophilus*-like Organism plus Swine Fever Virus and (d) Control Material

Experiment No.	*Inoculum* (*For details see "Materials and Methods"*)	*Pig No.* (*Fig. 1*)	*Days between inoculation and:-*		Haemophilus *recovered*	*Macroscopic pneumonia* (*See Fig. 1*)	*Pleurisy*
			Death	*Slaughter*			
	Naturally infected	1	—	18	+	+	+
Preliminary	Unfiltered lung suspension from Pig No. 1	2	—	14	—	+	—
		3	18	—	—	+	—
		4	25	—	+	+	+
1	*Haemophilus* from Pig No. 4 grown in pig serum broth	5	—	14	—	—	—
		6	—	21	—	—	—
		7	—	28	—	—	—
	Filtered lung suspension from Pig No. 4 (containing swine fever virus)	24	—	14	—	+	—
		25	17	—	—	+	—
		26	18	—	—	+	—
	Haemophilus from Pig No. 4 grown in pig serum broth plus filtered lung suspension from Pig No. 4	15	—	14	—	+	—
		16	17	—	+	+	+
		17	25	—	+	+	+
	Sterile pig serum broth	12	—	14	—	—	—
		13	—	21	—	—	—
		14	—	28	—	—	—
	Filtrate of *Haemophilus* culture in pig serum broth	22	—	14	—	—	—
		23	—	21	—	—	—
2	*Haemophilus* grown on the special medium	8	—	25	—	—	—
		9	—	25	—	—	—
		10	—	25	—	—	—
		11	—	25	—	—	—
	Filtrate of spleens from swine fever pigs	27	—	13	—	+	—
		28	20	—	—	+	—
		29	—	20	—	+	—
		30	—	20	—	+	—
	Haemophilus grown on the special medium plus filtrate of spleens from swine fever pigs	18	—	13	+	+	+
		19	15	—	+	+	+
		20	15	—	+	+	+
		21	19	—	+	+	+

Macroscopic appearance of the lungs. The distribution of macroscopic lesions in these animals is shown in Fig. 1, and one of the lungs, representative of them all, is shown in Fig. 2 (pig 26). The pneumonic areas were greyish-pink in colour, firm on palpation, and sank in water. They were indistinguishable from the lesions associated with "virus pneumonia" (Betts, 1952). There was no evidence of pleurisy.

Fig. 1.



The macroscopic appearance of lungs. The *Haemophilus*-like organism was isolated from pigs Nos. 1, 4, 16, 17, 18, 19, 20 and 21; pleurisy was present only in these animals. S/18, or D/18 = slaughtered, or died, 18 days after intratracheal inoculation.

Microscopic appearance of the lungs. Although the pneumonic areas were indistinguishable macroscopically from the lesions of "virus pneumonia", the histological picture was quite different. The most obvious lesion was collapse, varying in degree from complete obliteration of the alveoli to partial collapse with numerous histiocytes (Fig. 6). In some areas neutrophils were fairly numerous and sometimes they occurred in small, dense masses; neutrophils were commonly found in the bronchial and bronchiolar exudate and in this situation they had a degenerate appearance. Cells of septal type were usually numerous and occasionally they had two or

rarely three or more nuclei. The bronchiole epithelium frequently appeared hyperplastic (Fig. 6). Interlobular septa were frequently distended and lymphatic vessels were dilated. Small haemorrhages were present in some sections and occasionally cuffing of blood vessels with small round cells was noted (Fig. 7). There was no evidence of pleurisy in any section. This histological picture occurred in all the lungs examined.

Bacteriological examination of the lungs. A *Pasteurella* was isolated from pig 28, but neither the *Haemophilus* nor any other organism was isolated from the other six lungs; it seems unlikely, therefore, that pneumonic lesions in swine fever are necessarily associated with the presence of *Pasteurella suis*, as suggested by Davies (1949). It may be noted, further, that Nieberle and Cohrs (1931) have described a croupous pneumonia as being associated with swine fever, often accompanied by pleurisy and sometimes showing multiple areas of necrosis due, as the authors say, to various causes. In view of the findings recorded here in animals experimentally inoculated intratracheally with a suspension of pure swine fever virus in lung tissue, we conclude that the virus is capable of creating a pneumonia that has the constant histological features detailed above. Confirmation of this was obtained by finding small areas of pneumonia with the histological picture described above in three pigs inoculated intraperitoneally with a Seitz EK filtrate of lung suspension containing the swine fever virus.

Experimental Inoculations with the Haemophilus-like Organism plus Swine Fever Virus

In the preliminary experiment three pigs were inoculated with an unfiltered suspension of the original lung (i.e. with the *Haemophilus* plus swine fever virus), and in the other two experiments seven pigs received cultures of the organism plus swine fever virus (Table 1 and Fig. 1).

Clinical observations. The clinical symptoms shown by these animals were essentially similar to those seen in the pigs that received swine fever virus only; there was, however, the difference that the animals given the *Haemophilus* plus swine fever virus were more severely ill and in some cases there was a thick nasal discharge.

Macroscopic appearance of the lungs. All the lungs were extensively pneumonic, and in two cases, 19 and 21, there was involvement of the right diaphragmatic lobe. The distribution of the lesions is shown in Fig. 1 and the overall increase in pneumonic tissue when compared with pigs given swine fever virus only is clearly shown. In addition to pneumonia, there was pleurisy in seven of the ten cases, including the three pigs inoculated with unfiltered lung. It is of interest that subsequent bacteriological and histological examinations failed to show the presence of the *Haemophilus* in the three cases, 2, 3 and 15, in which pleurisy was absent (Table 1 and Fig. 1); pleurisy, therefore, was constantly associated with the

Haemophilus and did not occur in any animal inoculated with swine fever virus only. The macroscopic appearance of the lungs from one of the pigs inoculated with the *Haemophilus* plus swine fever virus is shown in Fig. 3 (pig 4); this lung was typical of all the seven cases from which the *Haemophilus* was isolated. The pleurisy is well shown. The most severely affected areas of lung in all these animals were dark, solid, and cut like liver; they were studded with vast numbers of tiny greyish nodules (Fig. 3). In some cases where the disease was well advanced, e.g. pig 17 that died 25 days after inoculation, the nodules had coalesced to form greyish areas of up to about half a centimetre in diameter. On section of a pneumonic area a creamy pus could usually be squeezed from the small bronchi. The nodular lesion was not present in any of the three pigs, 2, 3 and 15, from which the *Haemophilus* was not subsequently isolated. It appeared, therefore, that the macroscopic changes attributable to the *Haemophilus*-like organism as distinct from the swine fever virus—were (a) small, nodular foci (b) liver-like solidification (c) pleurisy.

Microscopic appearance of the lungs. Microscopic examination of the pneumonic areas under a low power showed multiple reactionary foci, often of oval shape and with a "mulberry" outline (Figs. 11 and 12). Many of these foci were invisible to the naked-eye, but others were easily seen when the section was held against the light. A higher magnification showed that these foci were composed of a number of small groups of cells that had a characteristic whorled appearance. At the centre of these cell groups there was a compact mass of Gram-negative organisms, and in the immediate vicinity of these there were deeply staining round cells (Fig. 8); outside the round cells—and responsible for the whorled appearance—there were deeply staining fibroblasts (Fig. 9). Examination of all sections indicated that the formation of these whorled cell groups was the earliest reaction to the *Haemophilus*-like organism; this reaction usually occurred within the lumen of an alveolus, but it was occasionally noted among the cells of the bronchial exudate. The larger reactionary foci, the biggest of which were visible to the naked-eye, were formed by the coalescence of many of these tiny whorled foci (Fig. 10). In the early stages of coalescence the foci were compact (Fig. 11) but later the centre of the developing nodule became more open and the alveolar lung structure was clearly visible (Fig. 12). The *Haemophilus*-like organism was constantly associated with the tiny whorls of fibroblasts, but was difficult to find elsewhere except in some animals where it occurred in clusters in the grossly abnormal pleura (Fig. 15). The lung tissue between the developing nodules usually contained much cellular exudate, mainly of round cells with moderate numbers of neutrophils; sometimes this tissue was collapsed, but in other areas the alveolar outline was clearly visible through the cellular exudate. The development of the characteristic nodules occurred rapidly after experimental inocula-

tion, for quite large nodules were found in the animal killed 13 days after inoculation (Fig. 1, No. 18). At an early stage in the growth of these nodules an extensive fibroblast reaction began to wall them off from the surrounding lung tissue (Figs. 13 and 14), and spread outwards to involve, in some cases, the interalveolar tissue and bronchioles in the immediate vicinity. The lung septa in the vicinity of the nodules were greatly thickened in all cases and their lymph channels were distended. This thickening began as an apparently oedematous distension, but later there was a very considerable development of granulation tissue. The pleura was grossly abnormal in all cases (Fig. 15), with fibrinous thickening and much granulation tissue in the deeper layers and was often studded with tight masses of the *Haemophilus*-like organism.

A variation in the reaction to the *Haemophilus*-like organism was seen in some areas of the lungs of pigs 19 and 20; in these areas the picture was of lobar pneumonia with a dense cellular exudate in which the organism was present in enormous numbers. It seems likely that this type of reaction was due to the presence of an overwhelming number of organisms in the alveoli, although there was evidence, here and there, of the beginning of the more typical fibroblast reaction.

The nodular lesion was only found in those animals from which the *Haemophilus*-like organism was isolated post-mortem. The *Haemophilus* was not isolated from pigs 2, 3 and 15 although they had been inoculated with it; no nodular lesions were found in these animals.

Bacteriological examination of the lungs. The *Haemophilus*-like organism was isolated from one pig of three inoculated with unfiltered material from the original lung and from six of the seven pigs inoculated with *Haemophilus* culture plus swine fever virus (Table 1). No other organism of known pathogenic significance was isolated from these lungs.

DISCUSSION

It has not been found possible to give a specific name to the organism described in this paper. It has been called "*Haemophilus*-like" because it appears to resemble most closely members of the genus *Haemophilus*. Culturally it does not appear to be identical with *Haemophilus influenzae suis* described by Lewis and Shope, and the organism with which Shope (1931 a and b) worked did not apparently produce the characteristic histological picture that we have described.

CONCLUSIONS

A *Haemophilus*-like organism, not apparently identical with *H. influenzae suis*, was isolated together with swine fever virus from a pneumonic pig lung.

The organism was a small, Gram-negative, pleomorphic rod

showing filamentous forms and evidence of capsulation. It was non-motile and catalase negative. Its colonial form was similar to *H. influenzae* and, like the latter, it showed satellitism when growing in approximation to *Staphylococcus aureus*. It did not, however, require factors X and V as described for *H. influenzae*, but did require a factor to be found in blood, serum, and commercial yeast extract.

Intratracheal injection of cultures of the organism failed to cause disease in any of seven pigs and the organism was not isolated from these animals when they were killed for examination 14 to 25 days after inoculation.

Intratracheal injection of unfiltered material from the original lung, or of cultures of the organism together with swine fever virus, caused pneumonia and pleurisy in seven of ten pigs; the organism was recovered from all these lungs except the three not showing pleurisy.

The pneumonia caused by intratracheal injection of swine fever virus only was characterised microscopically by collapse, with a varying degree of cellular exudate.

The pneumonia caused by intratracheal injection of swine fever virus plus the *Haemophilus*-like organism was characterised microscopically by the changes associated with swine fever virus plus a nodular lesion which is described in detail and was associated with the *Haemophilus*, and by extensive pleurisy.

ACKNOWLEDGMENTS

We are grateful to our Director, Dr. W. S. Gordon, C.B.E., for his interest in this work, to Mr. H. Broom for examining the nutritional requirements of the organism, to Mr. T. M. Doyle for supplying the crystal violet vaccine, and to Dr. J. H. Taylor for the lapinised virus vaccine and hyperimmune serum. It is also a pleasure to acknowledge our indebtedness to Mr. F. Summerfield, A.R.P.S. for preparing the photographs and to Mr. R. J. Taylor and Mr. P. F. Dennis for technical assistance.

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LEGENDS TO ILLUSTRATIONS

Fig. 2. Lung of pig 26, inoculated intratracheally with swine fever virus only.

Fig. 3. Lung of pig 4 inoculated intratracheally with the *Haemophilus*-like organism plus swine fever virus. Note pleurisy and the small nodular lesions in the right cardiac lobe.

Fig. 4. A 48-hour growth of the *Haemophilus*-like organism. Gram stain × about 900.

Fig. 5. Satellitism of the *Haemophilus*-like organism round a staphylococcal colony.

Fig. 6. Pneumonia attributable to swine fever virus. Note partial collapse, cellular exudate, and hyperplastic appearance of the bronchiolar epithelium. H & E stain × about 135.

Fig. 7. Perivascular cuffing in a lung infected with the swine fever virus only. H & E stain × about 400.

Fig. 8. The earliest lesion attributable to the *Haemophilus*-like organism. Small round cells clustered about the organism, apparently in an alveolus; fibroblasts surrounding the round cells. H & E stain × about 400.

Fig. 9. The next stage in the development of the reaction to the *Haemophilus*-like organism. Fibroblasts forming a whorl round a mass of organisms. H & E stain × about 400.

Fig. 10. Several fibroblast whorls, with compact masses of the *Haemophilus*-like organism, joining up to form a nodule. H & E stain × about 400.

Fig. 11. A nodule showing the characteristic "mulberry" outline. The fibroblast whorls are ranged round the outside, with the centre of the nodule beginning to open. H & E stain × about 135.

Fig. 12. The centre of the nodule is now open, with the alveolar lung structure visible. H & E stain × about 135.

Figs. 13 and 14. High and low power views of the rapidly developing granulation tissue that surrounds the nodule with a dense capsule. H & E stain. Fig. 13 × about 400: Fig. 14 × about 135.

Fig. 15. The greatly thickened pleura, with granulation tissue in the deepest layers. The dark line is composed of densely staining fibroblasts, and above this there are several compact masses of the *Haemophilus*-like organism. H & E stain × about 135.

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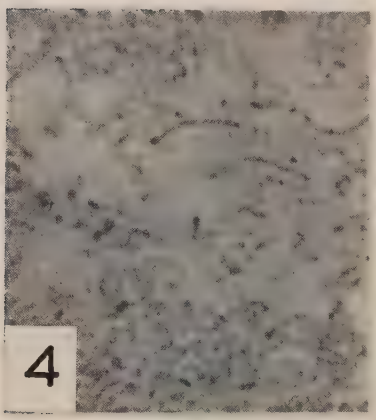
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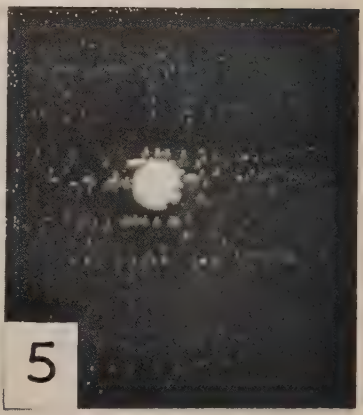
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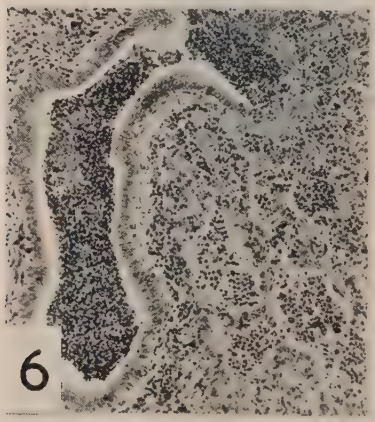
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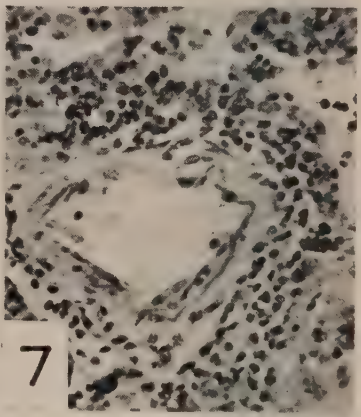
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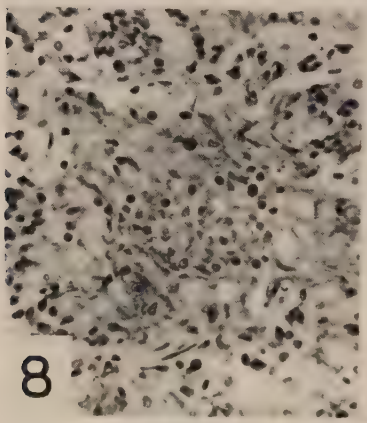
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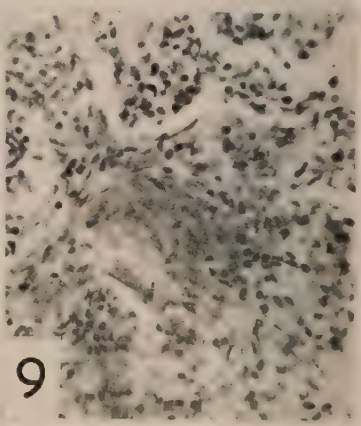
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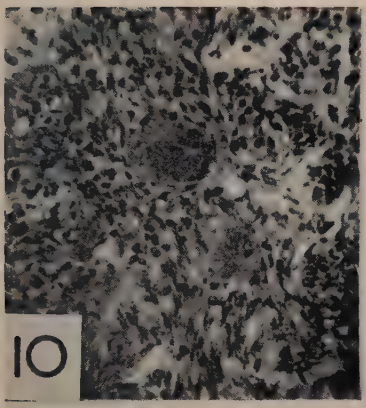
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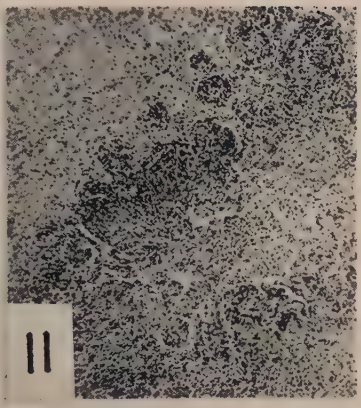
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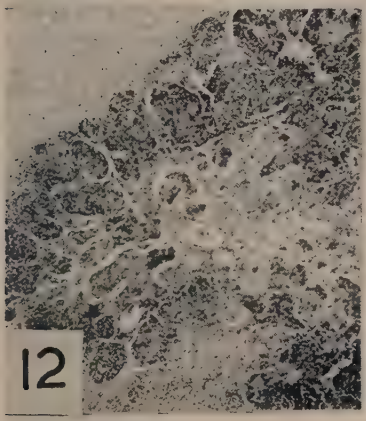
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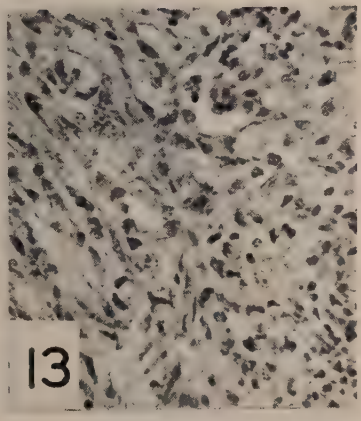
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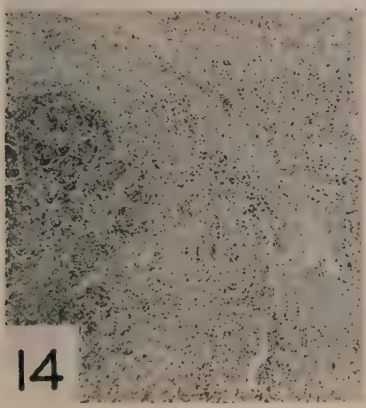
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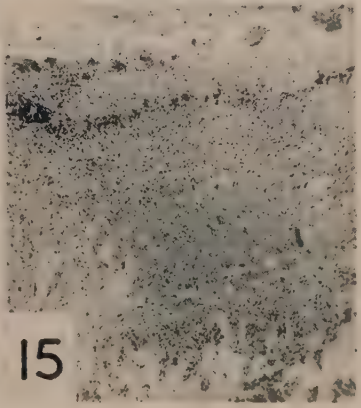
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SOME OBSERVATIONS ON GLUCOSE, KETONE BODIES AND VOLATILE FATTY ACIDS IN THE BLOOD OF DAIRY CATTLE

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INTRODUCTION

The determinations of blood glucose, ketone bodies and volatile fatty acids described in this paper were carried out simultaneously with the estimations of plasma 17-hydroxycortico-steroids described by Paterson (1957). It was intended to extend observations by earlier workers on changes in blood composition at parturition and in early lactation in normal cows. It has been reported that the blood level of glucose increases just before parturition, decreases immediately after parturition and thereafter is constant for one to four weeks (Godden and Allcroft, 1932; van Soest and Blosser, 1954). Knodt, Shaw and White (1942) and Sampson and Boley (1945) have reported that the blood level of ketone bodies shows no change at parturition, but is higher in early than in late lactation. No observations on blood levels of volatile fatty acids in pregnancy and lactation appear to have been reported.

Shaw (1943) studied the development of ketosis by estimation of blood glucose and ketone bodies in early lactation in cows from a herd with a high incidence of ketosis. In cows developing ketosis the decrease in blood glucose following parturition continued for four to ten days, during which time blood ketone bodies increased markedly, until clinical symptoms of ketosis appeared. It was intended to extend Shaw's work by observations on cows which had a history of ketosis in previous lactations and which might thus be expected to develop ketosis in the lactation under study. None of these animals developed ketosis in the period of study however and so this paper deals only with clinically normal animals.

METHODS

Blood glucose. Miller and van Slyke (1936).

Plasma volatile fatty acids. Conway and Downey (1950).

Blood ketone bodies. Blood ketone bodies were estimated by a modification of the method of Greenberg and Lester (1944). The reflux units described by these authors were found inconvenient in routine operation and instead the conversion of β-hydroxybutyric acid and acetoacetic acid to acetone was carried out in glass-stoppered test tubes heated in a boiling water bath. The stoppers, lubricated by a silicone grease, were retained in the tubes by a rubber-cushioned metal plate. The time of extraction of acetone 2:4 dinitro-phenylhydrazone with carbon tetrachloride was increased to 25 min. since 10 min. was found to be in-

sufficient for complete reaction. The 2:4 dinitro-phenylhydrazine reagent was washed with carbon tetrachloride to give lower blanks as recommended by Pennington (1954).

β-hydroxybutyric acid was at first estimated by difference from determinations of total ketones and of acetone and acetoacetic acid, but this procedure was eventually found to be unreliable at normal blood levels of these substances due to the inconsistent recovery of acetone and acetoacetic acid in the method for total ketones. The method described by Greenberg and Lester (1944) for the direct determination of β-hydroxybutyric acid was found to be reliable. Unfortunately most of the determinations of β-hydroxybutyric acid were made by the former method, and therefore no results from estimations of β-hydroxybutyric acid have been included in this paper.

RESULTS

In this and following sections plasma volatile fatty acids will be referred to as plasma V.F.A. The cows in this study were those used by Paterson (1957) and since all remained clinically normal throughout the period of study it seemed unlikely that any differences would be found between the normal cows and those with a history of ketosis in previous lactations.

Comparison of Normal Cows and Cows with Previous Ketosis

Since seasonal variations occurred in the blood levels of glucose, ketone bodies and V.F.A. in normal cows, the results from cows

TABLE 1

COMPARISON OF BLOOD LEVELS

Glucose, ketone bodies and volatile fatty acids in normal cows and cows with previous ketosis

	Prepartum November-April		*Postpartum January-May*	
	Cows with previous ketosis	*Normal cows*	*Cows with previous ketosis*	*Normal cows*
Glucose (mg.% blood) Mean ± S.E. (no. of results)	43·5±7·3 (37)	44·4±6·1 (37)	47·6±6·4 (45)	48·0±5·4 (59)
Sig. of difference	't'=0·64, P<0·6		't'=0·28, P<0·8	
Plasma V.F.A. (mg. acetic acid %) Mean ± S.E. (no. of results)	7·2±2·4 (38)	7·9±2·5 (40)	6·9±3·0 (44)	6·6±3·7 (59)
Sig. of difference	't'=1·28, P<0·3		't'=0·38, P<0·8	
Acetone and Acetoacetate (mg. acetone % blood) Mean ± S.E. (no. of results)	2·5±1·0 (38)	2·0±0·9 (39)	2·4±1·3 (46)	2·2±1·0 (61)
Sig. of difference	't'=1·94, P<0·1		't'=0·90, P<0·4	

with previous ketosis are compared only with those from normal cows during the same months, November to May. From Table 1 it can be seen that no differences in the blood levels of glucose, ketone bodies and V.F.A. were found between normal cows and cows with a history of ketosis in previous lactations. In the following considerations the results from cows with previous ketosis are therefore included with those from normal cows.

Changes before and after Parturition

The mean blood levels of glucose, ketone bodies and V.F.A. for 10 day intervals during the period of study are given in Table 2. The increase in blood glucose between 20 to 11 days and 10 to 0 days prepartum is significant ('t' $=2{\cdot}18$, $P<0{\cdot}05$) as is the decrease between 0 to 10 and 11 to 20 days postpartum ('t' $=2{\cdot}38$, $P<0{\cdot}05$). No other significant changes in blood glucose level were seen in the period of study.

While plasma V.F.A. show a lower level at 0 to 10 days prepartum and a higher level at 11 to 20 days postpartum, no significant difference was found between any two successive 10 day periods. Postpartum, the mean level of V.F.A. for the period 0 to 30 days ($7{\cdot}1 \pm 3{\cdot}3$ mg. per cent) is significantly higher than the mean level for the period 31 to 70 days ($5{\cdot}9 \pm 3{\cdot}0$ mg. per cent), 't' $=2{\cdot}63$, $P<0{\cdot}01$. No significant changes occurred at any time in the blood level of ketone bodies.

Thus the overall picture is that the blood level of glucose increases just before parturition, and decreases thereafter; plasma V.F.A. levels are slightly higher in the first few weeks of lactation than subsequently, and ketone body levels show no change in the first 10 weeks of lactation. However, while most animals showed such a picture, there was some variation among individuals, particularly in the postpartum period. The results for four individual animals (Fig. 1) show the extent of this variability. Of the 21 cows used in this study 14 showed an increase in blood glucose before calving followed by a decrease soon after calving (examples A, B and D, Fig. 1). Four cows showed no apparent change in blood glucose over the period of parturition (example C). There was some variation in the extent of the decrease in blood glucose after calving and in the constancy of the postpartum glucose level. Of the 14 cows showing a decrease in blood glucose soon after calving, 8 showed a decrease to levels below 45 mg. per cent glucose, in some cases the decrease being very striking as in example A. Following these lower levels there was an increase to a relatively constant level. In others, after the decrease at parturition, the blood level of glucose was constant (example B) or increased slightly. Of the 7 cows which showed no appreciable change in blood glucose around parturition 6 showed a transient decrease to rather low levels 3 to 5 weeks after calving (example C). Thus two-thirds of the individual

TABLE 2

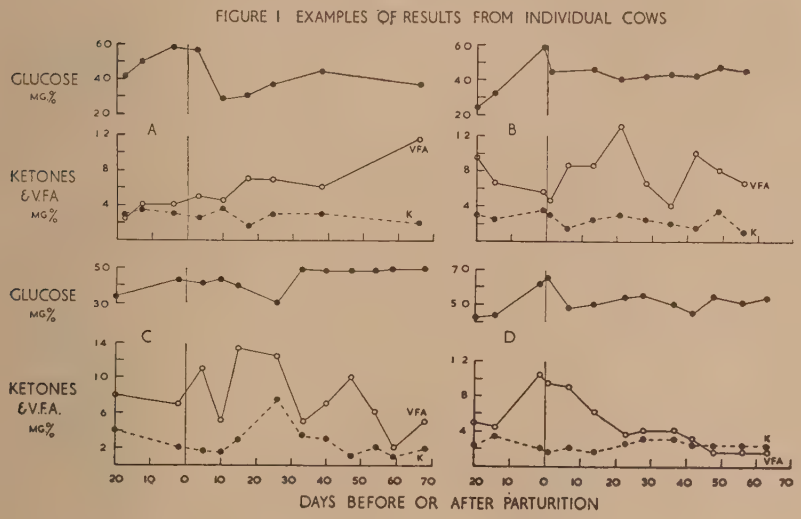
CHANGES IN THE BLOOD LEVELS

Glucose, ketone bodies and volatile fatty acids during pregnancy and lactation

	Days before parturition							*Days after parturition*						
	70-61	*60-51*	*50-41*	*40-31*	*30-21*	*20-11*	*10-0*	*0-10*	*11-20*	*21-30*	*31-40*	*41-50*	*51-60*	*61-70*
Glucose (mg. % blood) Mean ± S.E. (no. results)	46·6 ± 6·3 (11)	45·4 ± 4·5 (12)	44·2 ± 4·7 (13)	42·8 ± 4·8 (21)	44·5 ± 7·7 (18)	46·1 ± 6·1 (18)	52·4 ± 8·6 (27)	51·7 ± 9·8 (30)	46·1 ± 5·9 (29)	45·8 ± 7·8 (32)	48·1 ± 5·1 (24)	50·3 ± 16·8 (22)	48·1 ± 4·2 (21)	48·4 ± 7·1 (20)
Plasma VFA (mg. acetate %) Mean ± S.E. (no. results)	6·8 ± 1·7 (12)	6·6 ± 1·5 (12)	7·0 ± 2·3 (15)	7·3 ± 3·2 (21)	6·8 ± 2·8 (20)	6·5 ± 3·4 (19)	5·6 ± 3·0 (25)	6·7 ± 2·6 (31)	8·2 ± 3·9 (27)	6·6 ± 2·9 (32)	5·4 ± 2·6 (24)	5·9 ± 2·6 (23)	6·7 ± 3·7 (17)	5·7 ± 3·3 (19)
Acetone and acetoacetate (mg. acetone %) Mean ± S.E. (no. results)	2·0 ± 1·1 (12)	2·1 ± 0·7 (12)	2·1 ± 0·9 (15)	2·9 ± 1·2 (21)	2·7 ± 1·1 (19)	2·4 ± 1·0 (21)	2·7 ± 1·0 (27)	2·7 ± 1·1 (32)	3·2 ± 2·6 (29)	2·6 ± 1·3 (33)	2·6 ± 1·0 (23)	2·5 ± 1·7 (23)	2·3 ± 1·0 (21)	2·8 ± 1·7 (21)

cows showed a transient decrease in blood glucose to a moderately low or low level in the first ten weeks of lactation.

Fig. 1.



About half of the cows showed a decrease in the plasma level o V.F.A. before parturition. After calving, half of the cows showed transient high levels of V.F.A. (examples B and C, Fig. 1). Five of these showed a fairly well defined peak in V.F.A. (example B) but in six there was much fluctuation in the V.F.A. level (example C). Ten of these eleven cows showing high levels of V.F.A. postpartum showed also transient decreases in blood glucose; in six cases the lowest level of glucose was approximately coincident with the highest level of V.F.A., as in example C. Four cows showed a general increase in plasma V.F.A. throughout the postpartum period studied (example A) and four showed a general decrease (example D).

Only six cows showed any appreciable change in the blood level of ketone bodies, and these showed transient high levels approximately coincident with a high level of plasma V.F.A. postpartum (example C). No pattern of agreement between changes in blood levels of these substances and changes in milk yield or the level of milk yield could be seen.

Seasonal Variation

The results showing seasonal variation in the blood levels of glucose, ketone bodies and V.F.A. are given in Table 3. Both prepartum and postpartum the blood level of glucose was significantly higher in the period June to September than in other periods.

When considered by calendar months (Fig. 2), blood glucose is seen to be highest in July, to decrease progressively until December and then to increase slightly between January and March.

Fig. 2.

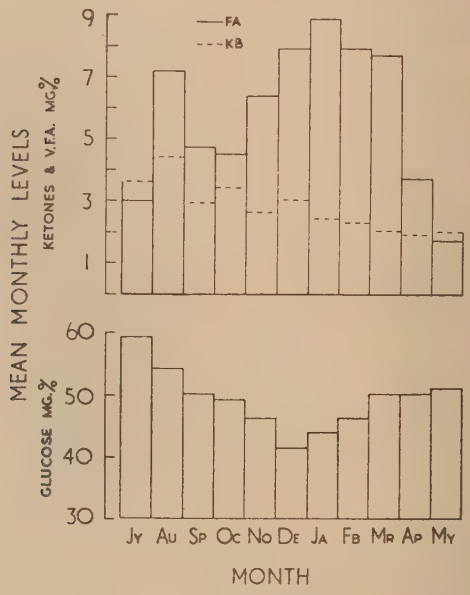


FIG. 2: SEASONAL VARIATION IN BLOOD COMPOSITION

The plasma level of V.F.A. prepartum was significantly lower in the period June to September than in the period October to December, and was lower in this period than in January to April. From Table 2 it would appear that the plasma level of V.F.A. postpartum was no different for the period July to September than for the period October to December. However among the cows studied in the period July to September was one which, although remaining clinically normal, showed very high levels of ketone bodies (14 mg. per cent) and V.F.A. (17 mg. per cent) for a short time. If the results from this animal are omitted from the results for the period July to September, the mean plasma level of V.F.A. is decreased to $4{\cdot}9 \pm 2{\cdot}3$ mg. per cent and while this is not significantly different from the mean for the period October to December, the overall postpartum picture now resembles more closely the prepartum picture. From Fig. 2 it can be seen that plasma V.F.A. levels are highest in the period December to March, i.e. approximately when blood glucose levels are lowest.

TABLE 3

SEASONAL VARIATION IN THE BLOOD LEVELS

Glucose, ketone bodies and volatile fatty acids.

	Prepartum			*Postpartum*		
	June-September	*October-December*	*January-April*	*July-September*	*October-December*	*January-May*
Glucose (mg. % blood) Mean ± S.E. (no. of results)	51·4±8·0 (23)	45·0±10·2 (48)	46·0±8·0 (50)	54·2±9·4 (30)	46·8±8·4 (51)	47·1±6·1 (92)
Sig. of difference	't'=2·61, P<0·02	't'=0·59, P<0·6		't' 3·69, P<0·001	't'=0·22, P<0·9	
Plasma VFA (mg. acetic acid %) Mean ± S.E. (no. of results)	4·5±2·4 (23)	6·4±2·7 (49)	7·6±2·3 (53)	6·1±3·8 (30)	5·9±2·4 (49)	7·3±3·2 (92)
Sig. of difference	't'=2·64, P<0·02	't' 2·52, P<0·02		't'=0·20, P<0·9	't' 2·79, P<0·01	
Acetone and Acetoacetate (mg. acetone % blood) Mean ± S.E. (no. of results)	2·9±1·2 (20)	2·9±1·5 (45)	2·0±0·9 (53)	3·9±2·5 (32)	3·0±0·7 (45)	2·2±1·1 (96)
Sig. of difference	't'=0·27, P<0·8	't'=4·73, P<0·001		't'=2·27, P<0·05	't' 3·40, P<0·01	

The prepartum blood level of ketone bodies was higher in the period June to December than in the period January to April (Table 3). The postpartum level was higher in July to September than in other periods. If the results from the cow showing rather higher than usual levels of ketone bodies and V.F.A., are excluded from the total results for this period, the mean for this period becomes $3{\cdot}2 \pm 1{\cdot}4$ mg. per cent which is little different from the mean for the period October to December. From Fig. 2 it can be seen that blood ketone body levels are lowest in the period January to April.

DISCUSSION

The determinations of plasma volatile fatty acids described in this paper represent an extension of observations by earlier workers on the composition of bovine blood in pregnancy and lactation. The method used gives good recoveries of formic, acetic and propionic acids which, according to Annison (1954), are the volatile fatty acids present in bovine plasma. It was not possible in the present work to confirm the identity of the acids estimated, but it is hoped to do so in subsequent work. Annison has found that of the total V.F.A. in bovine plasma 90 per cent is acetic acid and the values found by him (0·8 mM V.F.A. per litre = 4·8 mg. per cent acetic acid) are not greatly different from the mean value in the present work (6·6 mg. per cent acetic acid) although in the present work the normal range was found to be quite large (1 to 17 mg. per cent).

The seasonal variation in the blood levels of glucose, ketone bodies and V.F.A. in normal cows is of interest. A seasonal variation in the blood level of ketone bodies has been reported by Sampson and Boley (1945) who found higher levels in June and July than in November to January. These workers observed their animals only during these two periods whereas in the present work the period of observation was continuous from June to May. Our finding that blood ketone body levels are lowest in the months January to April is therefore not in conflict with the finding of Sampson and Boley. In contrast Knodt *et al.* (1942) have reported that blood ketone body levels are highest in winter, but they considered that the increase was due primarily to winter feeding of silage to the animals studied. Eden and Green (1940) reported that blood ketone body levels in a herd of 60 cows were higher in March than in July, a finding contrary to that in the present work and in the work of Sampson and Boley (1945). It is unfortunate that the ketone body levels reported in the present paper comprise only acetone and acetoacetic acid, but it is considered unlikely that the omission of β-hydroxybutyric acid would account for the discrepancy with the findings of Eden and Green.

No explanation can be offered for this variation in ketone body levels which is contrary to what might be expected from the seasonal

variation in blood glucose levels. That the blood level of glucose is lowest in winter (December and January) is in accord with the seasonal incidence of ketosis in this country, most cases occurring in the winter months (Allcroft, 1947). While the aetiology of ketosis is in some respects still obscure it is clear that the development of a hypoglycaemic condition is one stage in the development of ketosis, and it is possible that the low winter levels of glucose observed in this work indicate a tendency to the development of hypoglycaemia. It is unlikely that the seasonal change in blood glucose is related to the seasonal change in plasma 17-hydroxycorticosteroids reported previously (Paterson, 1957), for while the maximum blood level of glucose coincides with the maximum level of steroid in June and July, there the apparent correlation ends. There may be a relationship between the lower blood glucose in winter and the higher level of V.F.A. at that time, although there is nothing in the present work to show that one is the consequence of the other.

An increase in blood glucose before calving, and a decrease after calving were first reported by Godden and Allcroft (1932), and have subsequently been reported by Shaw (1943) and by van Soest and Blosser (1945). From the results given by Shaw the postpartum decrease in blood glucose can lead to transient lower levels of about 40 mg. per cent glucose, and from the results of van Soest and Blosser transient lower levels of 36 to 44 mg. per cent may be found soon after parturition. In the present work an increase in blood glucose level at parturition was seen in two thirds of the cows studied, and in the same proportion of animals there were seen also decreases postpartum to transient low levels of glucose, these lower levels ranging from 23 to 45 mg. per cent glucose.

The high levels of blood glucose at parturition may be related to the increase in plasma 17-hydroxycorticosteroids at that time (Paterson, 1957) for in most cows changes in blood glucose were parallel to changes in corticosteroids. Boddie (1935) however, detected lactose in the urine of parturient cows and suggested that the increased level of blood sugar at calving might be due to resorption of lactose from the udder.

No explanation can be offered for the transient low levels of glucose postpartum observed in the present work and by earlier workers. The rate and extent of the postpartum decrease in blood glucose in normal cows are, however, considerably less than in those cows developing ketosis observed by Shaw (1943). He found that the decrease in blood glucose just after parturition continued for 4 to 10 days, the final levels being 15 to 20 mg. per cent glucose, but in the present work a similar decrease was seen in only one cow (example A, Fig. 1) whose blood glucose level decreased from 57 mg. per cent to 29 mg. per cent in one week.

In the present work only three cows showed transient higher ketone body levels (5 to 6 mg. per cent acetone) associated with lower blood glucose levels. Shaw found that during the development

of a marked hypoglycaemia blood ketone body levels increased rapidly until clinical symptoms of ketosis appeared. It would appear that in most normal cows ketone body levels undergo no appreciable change.

The results presented in Table 2 and in Table 3 suggest that changes in blood glucose level might be reflected to some extent by changes in plasma V.F.A. level. In a few cases of ketosis where there was an opportunity for blood examination it was found that very high plasma levels of V.F.A. were associated with hypoglycaemia and ketonaemia (unpublished results). However while there might be some general relationship between the blood levels of glucose and V.F.A., the relationship is not seen in all cows. Ten cows showed both transient low levels of glucose and transient high levels of V.F.A. soon after calving but the changes were coincident in only five of them.

Only 16 normal cows and 5 cows with a history of ketosis were studied, and even within this small group a considerable variation between individual cows was seen. It is therefore probably unwise to make deductions from the results obtained in the present study. The seasonal variation observed in blood glucose, ketone bodies and V.F.A. deserves further study and an attempt made to determine its cause. It is of interest that in three or four cows a tendency was seen to what would usually be regarded as abnormal levels of glucose, ketone bodies and V.F.A., but further study of normal cows in early lactation is needed before it can be decided whether such findings represent the transient beginnings of an abnormal state, or merely the extremes of normal changes. Apart from these few cows, the lesser changes seen in most of the cows studied are worthy of further investigation, to see whether more frequent blood sampling would reveal a sequence of changes. If such studies are to contribute to our understanding of ketosis it is essential to extend Shaw's work by similar observations during the development of ketosis.

CONCLUSIONS

Determinations of blood glucose, acetone, acetoacetate and volatile fatty acids were made on 21 normal cows from 10 weeks before to 10 weeks after calving. In most cows blood glucose increased at parturition and decreased to transient lower levels after parturition. Volatile fatty acid levels were highest in the first few weeks of lactation, and in most cows ketone bodies showed no appreciable change.

Seasonal variation in the blood levels of these substances was seen, glucose being significantly lower in winter, volatile fatty acids significantly higher in winter, and ketone bodies lower in winter and early spring.

ACKNOWLEDGMENTS

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A CASE OF "THRUSH" (CANDIDIASIS) IN A PIGLET

By

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INTRODUCTION

Infection of the mouth and upper digestive tract by yeasts of the genus Candida (Monilia) is a well recognised condition in man and poultry. Apart from a short reference by Quin (1952) there are no records of its occurrence in the pig and so the following description of a single case was thought worthy of note.

GENERAL FINDINGS

Subject

One Large White x Wessex piglet, in a litter of eleven which were being reared artificially for a nutritional experiment, was affected. The piglets were weaned when 24 hours old and reared in individual cages on a semi-synthetic diet. (McCrea and Tribe, 1956).

History

When the piglet was fourteen days old it was noticed to have vomited a small amount of food material. By the eighteenth day vomiting was a frequent occurrence and the animal had become emaciated. On examination a white membrane was seen over the back of the mouth and root of the tongue. The piglet was destroyed.

Post-mortem Findings

The carcase was in very poor condition and somewhat oedematous. The major body organs showed no gross abnormality except for the alimentary tract. A white, glistening, irregular pseudo-membrane was present over the back of the tongue, the pharyngeal mucosa and the mucosa throughout the whole length of the oesophagus. In the terminal part of the oesophagus the membrane almost completely occluded the lumen, and on spreading out the incised organ, resembled a honeycomb (Fig. 1). The stomach contained a thin watery fluid, but the mucosa was not covered by deposit. The contents of the small and large intestine were also very fluid and in the caecum and colon were of a dark grey colour. The colonic mesentery showed a marked oedema. There was no evidence of a pseudo-membrane in any part of the alimentary tract posterior to the cardiac sphincter of the stomach.

Isolation of the Organism

Microscopical examination of films from the affected area having shown yeast-like bodies in addition to bacteria, cultures were prepared on suitable media at 27°C and 37°C. Since earlier

cases of severe diarrhoea in older piglets had revealed yeasts on culture from the colon (McCrea and Tribe, 1956) inoculation of plates was also made from this site. Plates incubated at the higher temperature yielded cultures of *B. coli* in 24 hours whilst those kept at 27°C showed typical yeast colonies after three days incubation. Microscopical examination having confirmed the typical morphology of yeasts, they were identified by Mr. P. K. C. Austwick who reported that the organism isolated from the oesophagus belonged to the genus Candida but had unfortunately died out before it could be accurately typed. The yeast isolated from the colon was *Candida albicans* and its presence there would seem to indicate a general distribution in the alimentary tract.

Histology

Sections of the oesophageal wall showed an unusual 'ballooning' of the stratified squamous epithelium (Fig. 2), apparently produced by infiltration of the epithelium above the stratum germinativum by mucus, neutrophils, yeast hyphae and degenerating cellular material which separated the basal from the superficial layers. The latter were extensively invaded by neutrophils and the cells were, for the most part, degenerate or necrotic. At intervals the basal and superficial layers were connected by long strands of vertically elongated epithelial cells. There was a moderate hypertrophy of the mucous glands of the submucosa and some degeneration of their secretory epithelium (Fig. 3). Pale staining fungal hyphae were seen in the large space between the epithelial cell layers and in the superficial layer of cells, and some oval yeast forms were also present. (Fig. 4). Overlying the superficial layer, long bacilli and filaments much narrower than typical fungal hyphae were present.

In sections stained by Gram all the organisms retained the purple stain in varying degree, but the centre of the large filaments was in places very pale, suggesting a failure to absorb both primary stain and counterstain. In sections stained with Periodic acid-Schiff the large filaments retained the pink stain whilst the narrower organisms did not. The latter were therefore assumed to be Lactobacilli although these were not detected on primary culture, probably because of insufficient incubation at 37°C.

DISCUSSION

The mycological and histological findings are very similar to those recorded in turkey poults by Blaxland and Fincham (1950) who did not, however, describe the unusual 'ballooning' of the epithelium which was a feature of this case. The source of infection could not be determined, but it seems probable that yeast spores were present in the building in which the pig was kept and it may be that, as in the case of poultry, yeasts are part of the normal flora of the upper digestive tract of the pig. Although it has been suggested that feeding rations supplemented with antibiotics might

encourage the development of fungi, we do not feel that this single case is sufficient evidence on which to support such a theory.

CONCLUSIONS

A case of "Thrush" occurring in a piglet being reared artificially is recorded and the pathology of the lesion is described and illustrated.

ACKNOWLEDGMENTS

We are particularly grateful to Mr. P. K. C. Austwick of the Veterinary Laboratory, Ministry of Agriculture, Weybridge for identifying the yeasts involved and to Mr. R. S. Undrill for the histological preparations and photographs.

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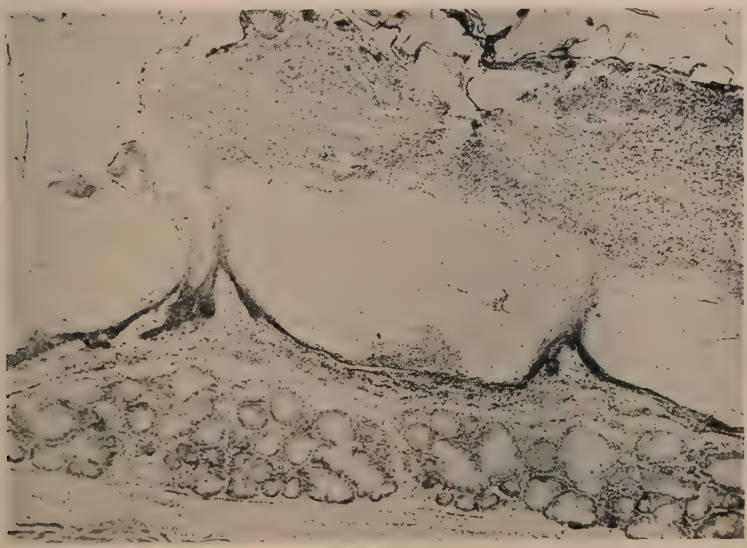
[*Received for publication, January 2nd,* 1957]

Fig. 1.

Oesophagus and stomach of piglet showing white pseudo-membrane over oesophageal mucosa.

Fig. 2.



Section of oesophageal wall showing 'ballooning' of epithelium. H. & E. × 36.

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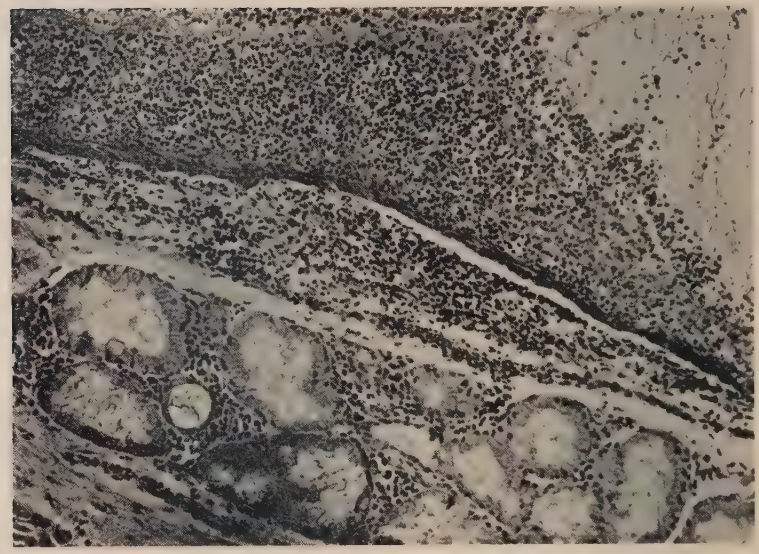
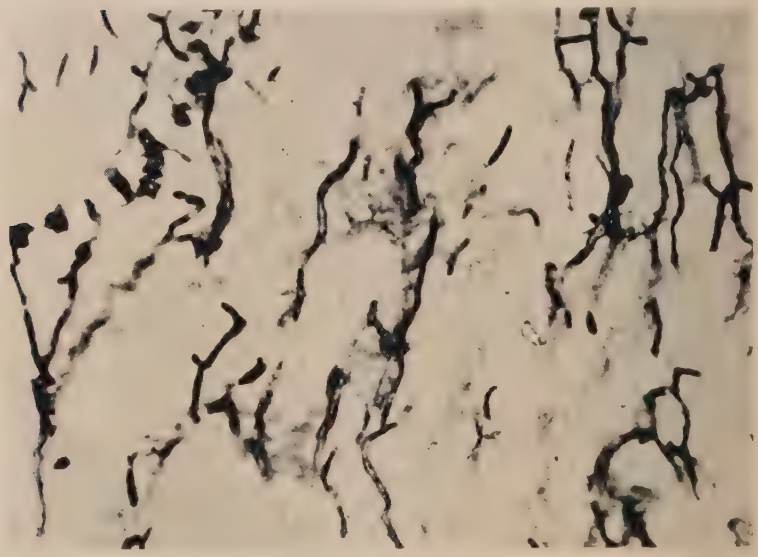
Fig. 3.



Cellular infiltration in mucosa and submucosa of oesophagus. H. & E. × 130.

Fig. 4.



Fungal hyphae showing irregular staining and yeast forms. Gram. × 560.

THE USE OF PAPER ELECTROPHORESIS AS AN AID TO DIAGNOSIS

By

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INTRODUCTION

The separation of serum proteins by paper electrophoresis has of recent years been employed widely in human medicine, but few reports have appeared in the veterinary literature and most of those are in German (Boguth, 1953; Chopard, 1954). It was therefore deemed of interest to report and assess its application to the domestic animals.

MATERIALS AND METHODS

Paper electrophoresis of serum. Strips of Whatman 3mm paper, 5 cm. x 25 cm. saturated with barbital buffer pH 8.6 (Flynn and Mayo, 1951) were supported in a horizontal type bath (E.E.L.*) and a current of 1·5 mA/strip passed for an hour. Serum (·01 ml.) was then applied and separation was allowed to continue for 16 hours. The strips were then dried in an oven at 120°C for 30 minutes.

Staining. The method of Jencks, Jetton and Durrum (1955) was used, except that the strips were only stained for 5 hours. Typical patterns for normal animals are shown in Plate 1.

Scanning. Dyed strips after soaking in liquid paraffin for 20 minutes were scanned with an automatic scanner (E.E.L.*).

RESULTS

Normal Animals

Serum Protein Values

The results obtained with the serum of different species of domestic animal are shown in Tables 1, 2 and 3. Not all sera from the horse show separation into α_1 and α_2 components. This was first reported by Chopard (1954) who also noted that when both were present α_2 was three times as large as α_1, a finding confirmed here. Boguth (1953) recognises 2 γ components in the cow. It has been found here that the γ globulin fraction is steeper and narrower than that reported by him. Occasionally it may be easily subdivided in the normal (Plate 1), but separation is far more apparent in certain pathological conditions, e.g. Johne's disease. Marked differences between immature pullets and laying hens were first described by Common, McKinley and Maw (1953) although quantitative evaluation was not carried out. It appears that great variation exists in the amount of β, and γ globulin present in laying birds. This may be related to the presumptive lipovitellin component (PLV) of McKinley,

*Evans Electroselenium Ltd., Harlow, Essex, England.

TABLE 1

		Albumen	α_1	α_2	β	γ
Horse (15 animals)	Rel. %	38·5	4·4	14·0	23·6	19·5
	S.D.	±3·38	±3·6		±3·46	±3·23

TABLE 2

		Albumen	α	β	γ
Cow (24 animals)	Rel. %	49·0	14·0	15·0	22·0
	S.D.	±6·65	±3·85	±3·91	±5·75
Sheep (15 animals)	Rel. %	52	6	18	24
	S.D.	±3·1	±1·07	±2·24	±3·2
Pig (10 animals)	Rel. %	40	18	17	25
	S.D.	±2·1	±2·01	±1·62	±2·45
Dog (17 animals)	Rel. %	47	12	14	25
	S.D.	±5·15	±1·58	±2·73	±4·11

TABLE 3

	Non-laying pullets, 15 birds	*Albumen*	α_1	α_2	α_3	β	γ
Hen	Rel. %	38·2	6·0	11·1	6·7	15·6	20·1
	S.D.	±4·1	±0·7	±1·9	±1·1	±2·1	±2·4
	Laying, 12 birds		α_1	α_{3+2}		β	γ
	Rel. %	23·5	4·3	21·2		20·7	31
	S.D.	±3·7	±0·4	±2·1		±4·3	±7·1

Oliver, Maw and Common (1953, 1954). It has been found that the anterior lipid fraction identified by these workers is associated with the α globulin fraction in both immature pullets and laying hens, but that with ovulation a great increase occurs in the PLV component associated with the β and γ globulin complex.

Six duplicate determinations were run on four different sera to assess reproducibility. Expressed as a percentage of total protein, reproducibility was in all cases within ± 4 per cent for albumen and ± 2 per cent for the globulin fractions. Sera which had been stored at —20°C for six months were tested against the original runs

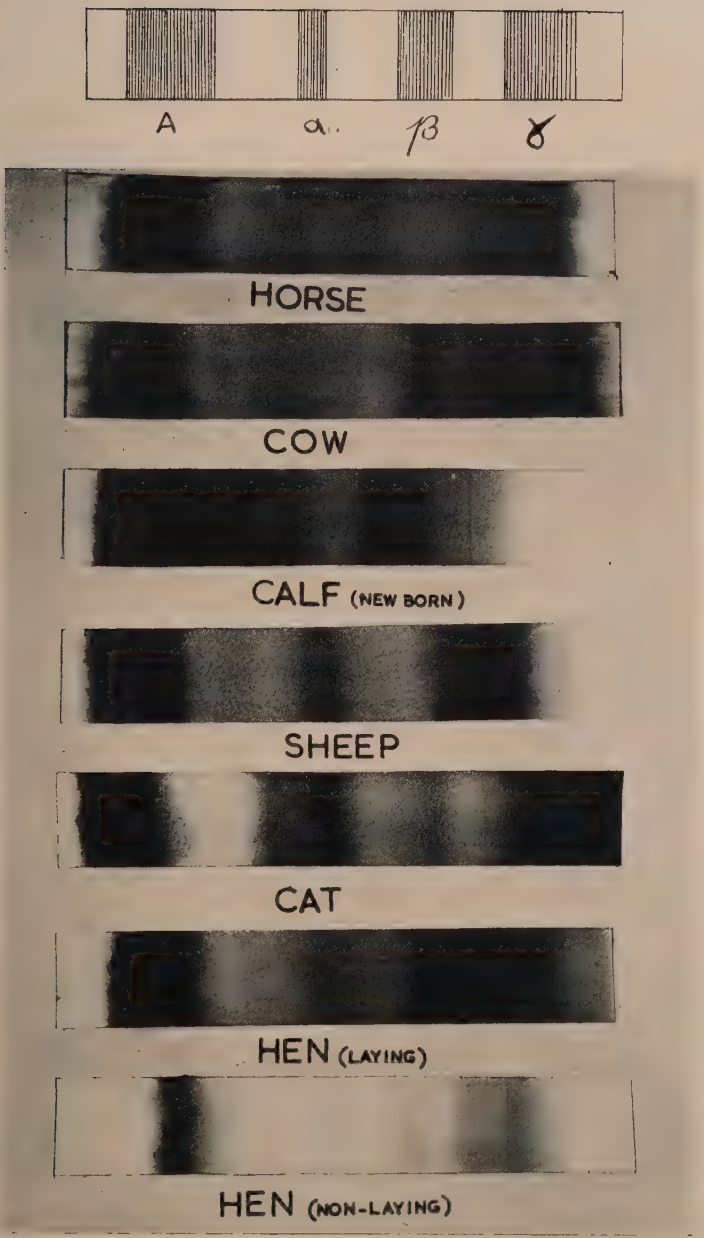

Plate 1. Serum protein patterns of the domestic animals stained with Brom-phenol blue.

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made shortly after collection. In no case were the values for the various fractions outside the range of normal reproducibility. Similar findings have been shown by Scudder (1940) using the classical Tiselius method.

Pregnancy

Boguth (1953) examined serum changes during pregnancy in the bitch and goat. Although alteration in pattern of the various fractions in the goat was indecisive, in the bitch a marked rise in α and β globulin occurred as gestation continued. It was of interest to determine if similar changes occurred in the sow. Results of fractionation of eight sera from pregnant sows are given in Table 4. It would appear that the sow, like the bitch, shows marked changes in the various fractions during pregnancy. Further work on this problem is continuing to ascertain if these findings are supported by a larger number of cases.

TABLE 4

SERUM CHANGES IN PREGNANCY

Rel. %	*Days after service*				*Service date uncertain*			*2 days after parturition*
	21	*35*	*45*	*58*				
A	38·5	34·5	29·5	28·8	34·5	39·2	29·2	30
α	16·1	17·2	22	22·2	18·1	21·9	28·6	22·4
β	19·2	24·4	27	28	25·8	22·2	20·4	25·5
γ	26	23·1	19·8	21	22·5	23·3	21·8	22·2
Sow	A	B	C	D	E	F	G	H

Serum Changes Associated with Disease

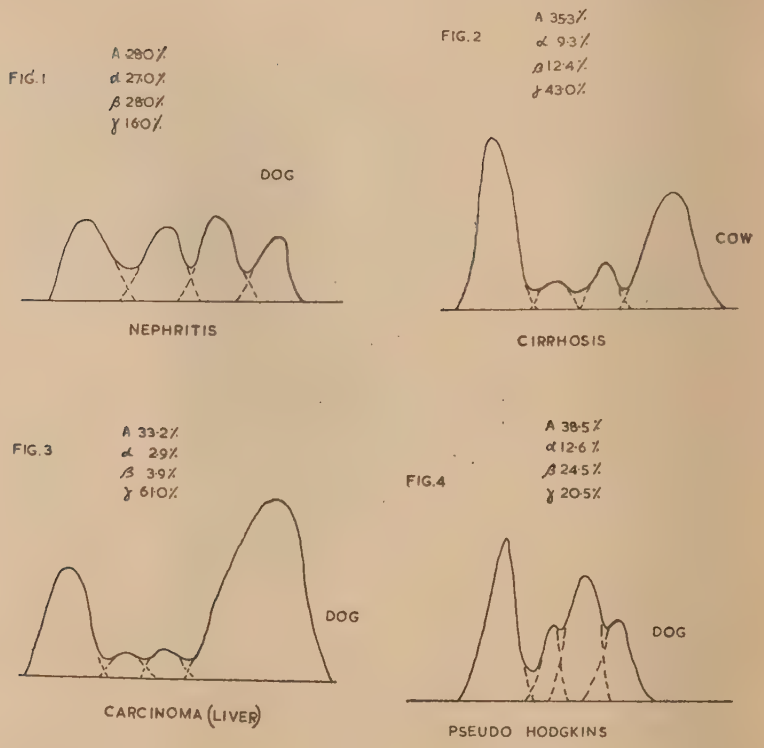
Certain syndromes present regular alteration of the serum protein pattern irrespective of species, e.g. nephritis and cirrhosis of the liver, while diseases specific to a species produce their own characteristic changes and serve as an aid to diagnosis. In all animals chronic infection is exemplified by an increased γ globulin.

Alterations which Occur Irrespective of Species

Nephritis. In chronic nephritis in the dog a marked decrease in albumen is present along with raised α and β globulins (Fig. 1). If marked oedema is present elevation of γ globulin is also seen, probably due to liver involvement.

In cases of vesical calculi showing no protein in the urine, and in chronic cystitis no change from the normal pattern was encountered.

Hepatitis. In hepatic cirrhosis marked increase in γ globulin with a decrease in the albumen fraction is found in all animals. Inconsistent small changes may be present in the α and β fractions,



(Fig. 2). Insufficient cases have been examined so far to enable differential diagnosis of the various forms of liver dysfunction to be assessed by paper electrophoresis. Results have demonstrated, however, that carcinoma of the liver causes a greatly increased γ globulin with reduction in the α and β fractions, (Fig. 3). Despite these findings liver function tests may be negative if under fifty per cent of the liver is involved. When attempting to assess prognosis it has been found that while the severity of liver damage is broadly related to the increase in γ globulin, this is only true up to a point, and the overall clinical picture must always be taken into consideration. Cases re-examined after several weeks have shown little change in the altered protein pattern although a marked worsening of the general condition was seen clinically.

Tumours. Boguth (1954) reported changes in α, β and γ globulins in cases of malignant tumour. Results from numerous cases confirmed by post-mortem or biopsy indicate that the greatest changes occur in the β and γ globulin fractions; abnormal splitting of these fractions may also be present. No relationship was apparent between the changes in these fractions and the type of tumour or severity of its effects e.g. tumours of rib and prostate have both been found to give increased γ globulin while a Sertoli cell tumour gave a normal protein pattern. An example of increased β globulin is seen in (Fig. 4) taken from a Terrier with Pseudo-Hodgkin's disease.

Changes Peculiar to a Species

Ruminant. In the ruminant, bacterial infections whether acute or chronic are represented by an increased γ globulin with a corresponding decrease in albumen. Boguth (1954) claimed that a change in α and β globulin was only seen in acute infection in the pig and dog, but it has been found here that virus infections in cattle and sheep are represented by alterations in these fractions with little change in γ globulin. Table 5 gives figures found in virus pneumonia in the calf and a case of Jaagziekte in sheep. Bacterial infection is shown by Case 3 from a markedly emaciated cow on which liver function tests (dye excretion and flocculation tests) gave normal values, but at post-mortem multiple abscesses caused by *C. pyogenes* were found in the abdominal cavity.

TABLE 5

	Albumen	α	β	γ	*Total protein*
Virus pneumonia (Calf)	33*	19·8	26	21	5·8g.
Jaagziekte (Sheep)	37	α_1 4·3 α_2 17·4	15·2	26·5	7g.
C. pyogenes (Cow)	23·7	12·4	15·1	49	6·1g.

*All figures give relative %.

The problem of tuberculosis has been investigated by Boguth (1954), Zimmerli (1955) and Weber (1955). In animals which although positive to the tuberculin test showed no clinical symptoms, no change was found, but where clinical signs were evident, a rise in γ globulin and diminution in the other globulins occurred. In markedly affected animals, the rise in γ globulin was sufficiently great to permit diagnostic identification.

In applying these observations to Johne's Disease equally rewarding results have been obtained. In all confirmed cases examined, a hundred per cent rise has been shown by the γ globulin, and usually this fraction is split into two components. The criteria of infection

have been the post-mortem appearance of the gut, the microscopic identification of Johne's bacillus and a positive reaction to Hole's test (1952). In one case, while the first two conditions were present Hole's test was negative. Table 6 gives figures for four typical cases and for comparison, results from three bullocks showing scouring with biochemically established copper deficiency.

TABLE 6

Johne's disease				*Copper deficiency*			
Albumen	α	β	γ	*Albumen*	α	β	γ
33·5	19·3	12·5	43·8	48	15·6	14·6	21
35	10·9	9·6	45·2	47	17·4	15·4	24·5
21	18·8	18·2	42	53	13·4	15·8	19·4
36·5	13·1	11	39				

Pig. In this animal acute infection results in an elevation of the α globulin fraction, more chronic infections give increases in β and γ globulins and transition stages may be represented by elevation in all globulin fractions with decreased albumen. Fig. 5 is an example of virus pneumonia in a young pig. Vesselinovitch (1955) submitted sera from young pigs suffering from bowel oedema to electrophoretic examination and at the same time conducted a study of normal values for this species at the age of 2 to 3 months. Cases presented here have not shown the fall in albumen or the rise in γ globulin reported by him, but confirm the elevation of α globulin. As the rise in α globulin was more marked in our cases, all of which showed staggering gait and oedema of the eyelids, it may be that samples were taken in a different phase of the disease. The sharp α globulin elevation supports the findings of Timoney (1956) that an haemolytic organism from the gut can be incriminated. Table 7 gives mean figures from affected piglets from four different farms.

TABLE 7

SERUM FRACTIONS IN BOWEL OEDEMA

Albumen	α	β	γ
37	40	13·6	8·1
37·5	42·5	14·6	6·3
36·5	30	20·5	7·5
37	37	18	9·2

Dog. Difficulty was experienced in classifying the type of response in the dog to a particular infection, probably due to the uncertain knowledge of the duration of the disease and the form of re-

FIG. 5

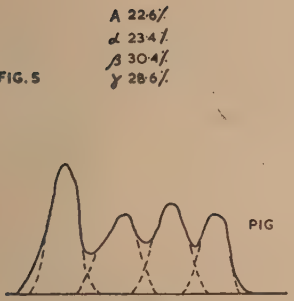


FIG. 6

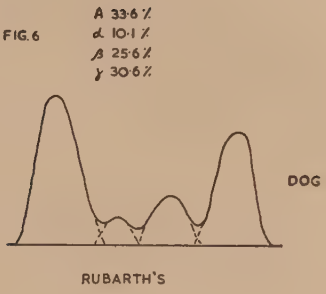


FIG. 7

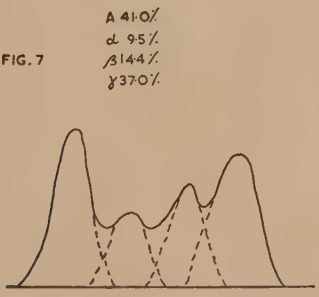


TABLE 8

SERUM FRACTIONS FOUND IN PYOMETRA IN THE DOG

Albumen	α	β	γ
33	13·8	26	28
27	10	22	40
21	15·1	27·5	29
31·5	13·5	24	31
28	27	14·2	29
28	28·5	13	31

sponse by the host, but it has been impossible to confirm Boguth's finding of a rise in α globulin in acute infection. In diseases considered acute, such as Distemper and Rubarth's Disease, (Fig. 6) a marked rise in β globulin was usual, and in distemper terminating with nervous upset or hard pad with keratosis, the rise in γ globulin was predominant. Fig. 7 is from a bitch suffering from fits following

a diagnosis of distemper two weeks previously. Boguth's claim that closed pyometra in the bitch is characterised by a rise in β globulin has been substantiated. His claim that a concomitant rise in α globulin denoted peritonitis with poor prognosis is of interest. Peritonitis with pyometra has not been observed here, but a number of open discharging cases have shown increased α fractions with normal or decreased β globulin (Table 8).

DISCUSSION

When assessing a novel method of investigation, consideration must be given to the time and work required and the specificity of the results, compared with existing methods used to support tentative clinical diagnosis.

With paper electrophoresis, once the apparatus is acquired the method is simple and reliable results can be achieved in a short period by unskilled personnel. Disadvantages are that the results are not available for at least 24 hours; further the changes in the protein fractions are not specific to any clinical condition so that their interpretation must always be made against the background of the full clinical picture. Despite these limitations paper electrophoresis may be of great value to the clinician in assessing the severity of a condition as reflected by changes in plasma proteins, and in following recovery or progress of an illness. In the differential diagnosis of Johne's disease, or in suspected liver dysfunction where specific test are still unsatisfactory, it has proved as reliable as existing methods. In pyometra the time taken to produce results means that present techniques are still the method of choice.

In its principal role as a simple method for following changes in the behaviour of plasma proteins in disease, paper electrophoresis has great advantages over the salting out method and the time-consuming classical electrophoretic procedures.

CONCLUSIONS

The values of the various serum protein fractions of normal domestic animals obtained by paper electrophoresis are given, and the changes encountered in various diseases are described.

These changes are for the most part non-specific and their interpretation can only be made with full knowledge of the clinical picture.

Paper electrophoresis is of great value in following the course of a disease, and for investigating changes in the serum fractions resulting from infection.

ACKNOWLEDGMENTS

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BLOOD CHANGES IN PIGLETS ASSOCIATED WITH WEANING

By

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INTRODUCTION

The period immediately subsequent to weaning has been considered to be the time of highest susceptibility of young pigs to bowel oedema (Timoney, 1950). It was thought possible, on the analogy of post-parturient paresis, that blood changes observed to occur in bowel oedema might represent an exaggeration of a normal variation at weaning. Interest centred upon serum proteins and electrolytes since a hypoproteinaemia had been reported in oedema cases by Luke and Gordon (1950) and disturbances in serum potassium levels appeared to be of some significance (Garner, unpublished data). A sudden change in diet has often been invoked as a contributory factor in the development of bowel oedema: the effects of weaning on to foods of widely differing protein content and subsequent interchanging of the diets were therefore studied.

METHODS

Animals. A litter of 12 Saddleback-Large White cross piglets born on the Langford House Farm on 20th March, 1955 was used. For the purposes of the experiment they were ear-marked A_{1-12} (A_{12} being the runt of the litter) and divided into three groups, Group 1 containing A_{1-4}, Group 2, A_{5-8} and Group 3, A_{9-12}.

Diets. From the time of beginning the experiment to the time of removal from the sow all the piglets were allowed access to a proprietary sow weaner meal containing 17 per cent protein. Water was provided *ad lib.* At weaning, Group 1 was retained on this diet, and Group 2 was given the same ration with the protein content increased to 26 per cent by addition of $\frac{1}{4}$ cwt. fish meal to each 1 cwt. of weaner meal. The third group received cooked kitchen swill (estimated to contain less than 5 per cent protein), supplemented when necessary through lack of sufficient swill with the weaner meal given to Group 1. The diets of Groups 2 and 3 were subsequently interchanged at 73 to 74 days as shown in the accompanying figure, Group 3 being maintained on the high protein ration until the termination of the experiment. The second group of animals was returned to this diet when 89 days old.

Blood samples. The piglets were bled by puncture of the anterior vena cava. No difficulty was usually experienced in carrying out this procedure and no untoward effects were observed.

Biochemical methods. Total and differential proteins were determined by a micro-Kjeldahl procedure, urea by means of urease (King, 1951),

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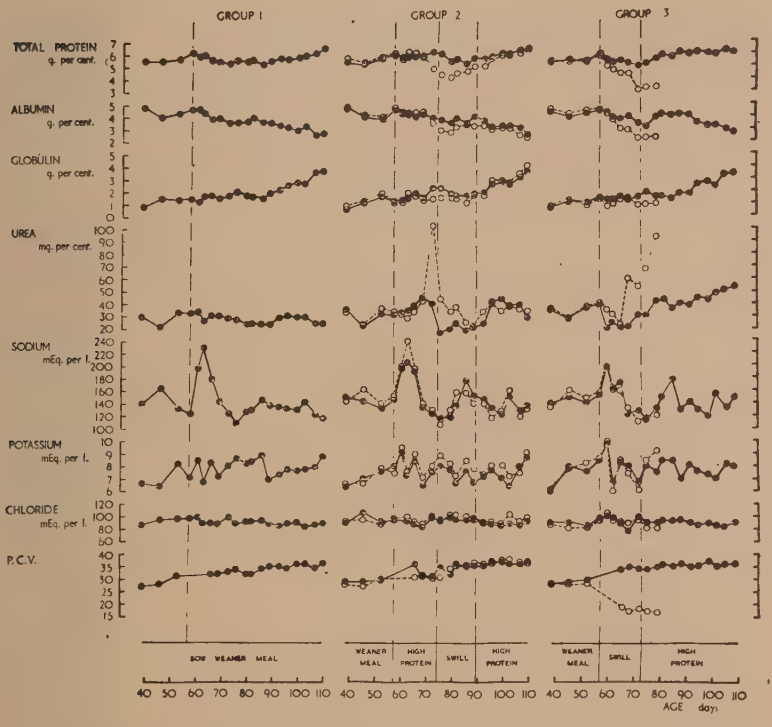
sodium and potassium by flame photometry, and chloride by the titration method of Schales and Schales (1941). All procedures were carried out on serum.

Fig. 1.



Changes in various blood constituents with age and change of diet. Group 1, means of A_{1-4}, ●—●; Group 2, means of A_{6-8}, ●—●, A_5, O---O; Group 3, means of A_{9-11}, ●—●, A_{12}, O---O. The vertical broken lines indicate weaning (at 57 days) or changes in diet (at 74 and 89 days in Group 2 and at 73 days in Group 3).

RESULTS

Mean values for total and differential serum proteins, urea, sodium, potassium, chloride and packed cell volume (used as a rough measure of the degree of haemoconcentration) are given in the accompanying figure. The mean values for Group 1 have been calculated from the results obtained from pigs $A_{1\,4}$, for Group 2, from A_{6-8} and for Group 3, from A_{9-11}. A_5 has been excluded because of its obviously abnormal behaviour with respect to serum urea levels. A_{12} was slaughtered when 112 days old: it was emaciated, jaundiced and unable to stand. At autopsy a small

amount of fluid was found in the abdominal cavity and the liver was bile-stained and friable. The serum contained 1·3 mg. per cent direct-reacting bilirubin and 2·1 mg. per cent indirect-reacting bilirubin.

Certain trends apparently unrelated to weaning or to the composition of the diet are obvious from the accompanying graphs. These include an increase with age in total protein and in the globulin fraction of serum, a decrease in the albumin fraction and an increase in the packed cell volume. Similar changes with age in serum proteins (Garner, 1952) and in the packed cell volume (Garner and Unsworth, 1953) have been described in cattle.

Weaning was followed in all three groups by an abrupt but transient increase in the serum sodium level. This may have been due in part to a sudden decrease in the water intake for it was least marked in Group 3 in which there was a concomitant fall in food (and protein) intake, i.e. in those animals in which there was least competition between sodium and urea for urinary excretion. A secondary rise followed some 2 to 3 weeks after weaning: this was least obvious in the group maintained on the same diet throughout. Fluctuations in the potassium concentration were apparent over the whole period of the experiment. The mean value for all groups (7·6 mEq./l., range 5·8 to 10·0 mEq./l.) was much higher than that usually associated with mammalian blood. Factors such as haemolysis which might have introduced grave errors in potassium determination were carefully controlled and it is thus not possible to dismiss the results as artefacts. The chloride concentration, in contrast, remained steady, varying slightly about a mean value of 112 mEq./l. for all groups.

Further changes in the diets of Groups 2 and 3 at 74 and 89 and at 73 days respectively had little apparent effect on the biochemical behaviour of the animals; the only blood constituent studied which appeared to vary directly with the protein content of the diet was urea. This is to be expected. Mean values for urea levels on different diets in terms of urea per cent were: Weaner meal 28 $\pm$4·6 mg.; High protein 40 $\pm$8·0 mg.; Swill 24 $\pm$6·8 mg. The differences between these means are highly significant. The very high serum urea concentration (106 mg. per cent) in pig A_5 while on the high protein regimen was not associated with clinical symptoms of any kind. It is interesting to note that in this animal and in A_{12}, an increase in serum urea was accompanied by a fall in both protein fractions, perhaps indicating that there was some diversion of amino-nitrogen from protein synthesis.

There appears to be little significance in any of these findings from the point of view of the aetiology of bowel oedema. It may be mentioned that an overall picture essentially similar to that described in Group 1 has also been observed in a litter of 4 piglets born on a farm on which bowel oedema was endemic and brought to this establishment prior to weaning.

CONCLUSIONS

Changes in serum proteins, urea, sodium, potassium and chloride and packed cell volume were studied in a litter of 12 pigs from the age of 38 to 109 days.

Serum total protein and globulin and packed cell volume increased with age and serum albumin decreased.

Weaning was followed by a well-marked but transient increase in the serum sodium concentration.

Sudden changes in the diet subsequent to weaning appeared to have no effect on any of the blood constituents studied other than urea: the urea concentration in the serum of the young pig is directly related to the protein content of its diet.

ACKNOWLEDGMENTS

We wish to thank Mr. K. England for technical assistance.

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COMPARATIVE COAGULATION STUDIES ON HORSE AND HUMAN BLOOD

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INTRODUCTION

Bell, Tomlin and Archer (1955) reported that normal horse plasma, when compared with normal human plasma, was deficient in anti-haemophilic globulin. This naturally occurring coagulation "defect" in the horse was similar to that known to exist in human classical haemophilia (haemophilia A). More recently, Sjølin (1956) has reported that the reduced thromboplastic activity of horse plasma was due to a deficiency of the Christmas factor (P.T.C. or plasma thromboplastin component) and that the anti-haemophilic globulin activity was not affected. In an attempt to find the reason for the discrepancy in the results of these two studies, the problem was re-investigated and extended.

MATERIALS AND METHODS

Collection of blood. Blood was collected from the external jugular vein of horses and ponies by clean venepuncture using paraffined syringes. Human blood was collected from an antecubital vein. It was then placed in siliconized bottles or tubes containing one tenth volume of 3·8 per cent trisodium citrate (w/v). The contents were mixed by gentle inversion several times.

Platelet counts were performed on whole blood by a direct counting method using a diluting fluid composed of 1 per cent sodium sequestrene in physiological saline containing a few crystals of brilliant cresyl blue.

Plasma prothrombin activity, was determined by the method of Quick (1939). Both human and horse brain thromboplastin were used. With this method fresh human plasma clots in 12 to 14 seconds when activated by human brain thromboplastin. The activity was also measured with Russell's Viper Venom (Stypven, Burroughs Wellcome) at a concentration of 1:10,000 as thromboplastin (with and without the lipid co-factor). The preparation of the lipid co-factor is described in the thromboplastin activity test.

Serum prothrombin activity. The method was essentially similar to that used for plasma prothrombin activity. Since the system did not contain fibrinogen, this was added to it. The materials were added strictly in the following order to the clotting tube: 0·1 ml. fibrinogen, 0·1 ml. thromboplastin, 0·1 ml. 0·025 M calcium chloride, and 0·1 ml. serum. The serum activity was estimated within two hours of the coagulation of the whole blood at 37°C.

Thromboplastic activity was measured by a slight modification of the method of Biggs and Douglas (1953); a chloroform extract of acetone dehydrated brain (both human and horse) prepared according to the method of Bell and Alton (1954) was used as a platelet substitute. This modification allows the use of a stable material of constant concentration and activity and overcomes the technical difficulty encountered in the preparation of platelet suspensions of uniform activity. The lipid material was used in an initial concentration of 0·25 mg./ml. In the test, the plasma and serum were diluted with imidazole buffer (pH 7·4). The brain lipid was diluted with physiological saline. Assays were done on the day of collection of the blood and also after storage of the plasma and serum for 24 hours at —25°C. Serum thromboplastic activity becomes maximal after a period of storage and thawing. Human and horse platelet—poor plasma was used as substrate.

Platelet thromboplastic activity was assayed by the method of Biggs and Douglas (1953). The separated platelets were washed three times with physiological saline.

Thrombin generation was measured by the method of Pitney and Dacie (1953). While this test measures, in a relatively simple way, the thromboplastic activity of blood, the results were found to be much less reproducible than those obtained with the thromboplastin generation test of Biggs and Douglas (1953). The test system consisted of 0·4 ml. platelet rich animal plasma + 0·1 ml. imidazole buffer or 0·1 ml. of the serum or plasma tested for their correcting activity.

Fibrinogen was dissolved in equal parts of imidazole buffer (pH 7·4) and physiological saline to a concentration of 400 mg./100 ml. It was prepared from human plasma by the method of Kekwick and Mackay (1954) and supplied by the Lister Institute of Preventive Medicine, London.

RESULTS

The blood from eight Thoroughbred horses and four Shetland ponies was studied.

Platelet Concentrations.

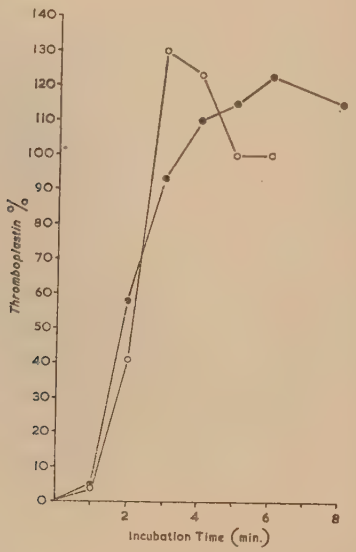
In comparison with normal human values, (200,000 to 400,000/mm.^{3}), the horses had moderately reduced whole blood platelet counts while the pony counts were within the human range (Table 1).

Plasma Prothrombin Activity

Prolonged clotting times were found in both the horse and pony plasmas when human brain was the source of thromboplastin. When horse brain thromboplastin was used the times were reduced by approximately one half (Table 1). The prothrombin time of human plasma in combination with horse brain thromboplastin instead of human brain was similarly affected (prolongation of the clotting time from 14 seconds to 25 seconds). This finding emphasised the importance of using the homologous species thromboplastin when estimating the plasma prothrombin times. In the

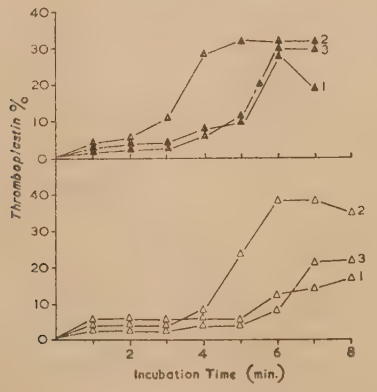
factor is also present in serum, and since horse serum is about as active as human serum in thromboplastic activity, a significant deficiency of P.T.A. appears to be excluded.

Fig. 1.



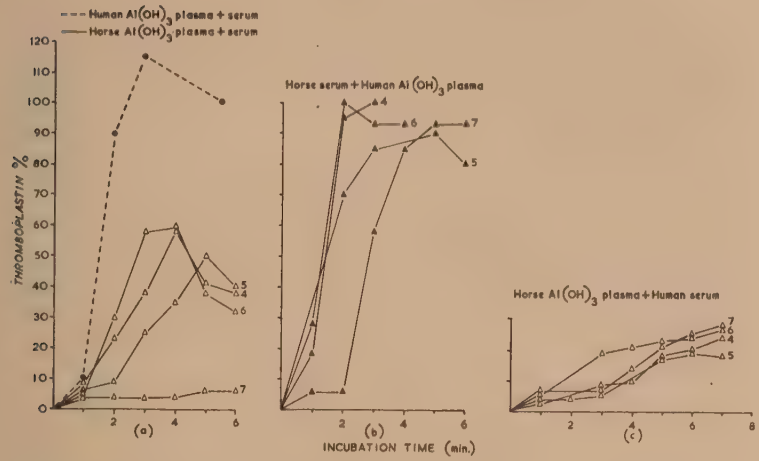
The thromboplastic activity of normal human plasma and serum.
○——○ Assayed with human brain lipid and human substrate plasma.
●——● Assayed with horse brain lipid and horse substrate plasma.

Fig. 2.



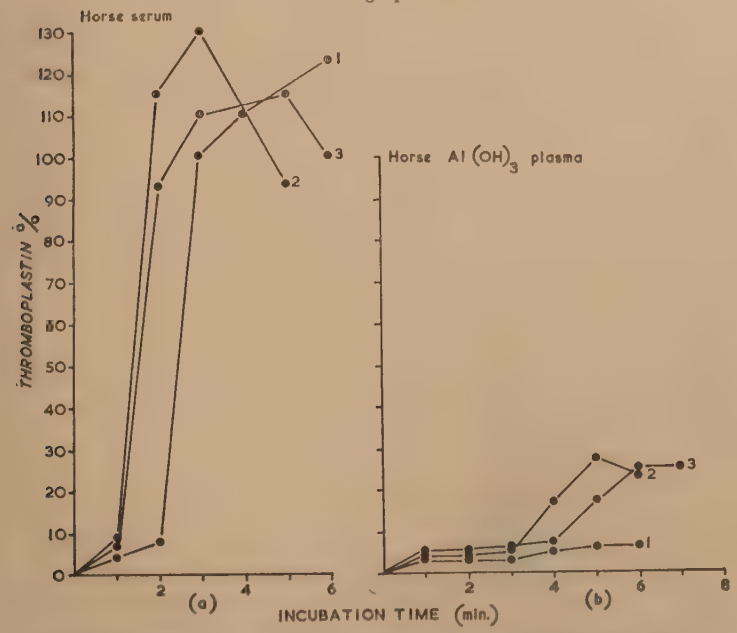
The thromboplastic activity of three normal horses (Nos. 1, 2 and 3).
▲——▲ Assayed with horse brain lipid and horse substrate plasma.
△——△ Assayed with human brain lipid and human substrate plasma.

Fig. 3.



The thromboplastic activity of the serum and plasma of four horses (Nos. 4, 5, 6 and 7).
(a) The homologous systems.
(b) Horse serum combined with human plasma.
(c) Human plasma combined with horse serum.

Fig. 4.



The thromboplastic activity of the serum and plasma of three horses (Nos. 1, 2 and 3).
(a) Homologous systems.
(b) Human plasma combined with horse serum.

TABLE 2

THE RESULTS OF THE THROMBIN GENERATION TESTS IN 2 PONIES AND 2 HORSES SELECTED AT RANDOM

	Fibrinogen clotting time (sec.) Incubation time (min.)									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
Pony 1. Plasma (control)	>60	>60	>60	>60	>60	41	27	24	22	23
Pony 1. Plasma+ Haemophilia A plasma	>60	>60	>60	>60	>60	60	35	20	18·5	16
Pony 1. Plasma+ Haemophilia B serum	>60	>60	53	28	19	14	14	12	12	18
Pony 1. Plasma+ Normal human serum	>60	>60	31	18	14	10	11			
Pony 2. Plasma (control)	>60	>60	>60	>60	>60	45	25	20	17	15
Pony 2. Plasma+ Haemophilia A plasma	>60	>60	>60	>60	45	23	16	12	15	
Pony 2. Plasma+ Haemophilia B serum	>60	>50	36	22	17	16	16	18		
Pony 2. Plasma+ Normal human serum	>60	45	27	20	16	17	13	18	29	
Haemophilia A plasma (control)	>60	>60	>60	>60	>60	>60	>60	>60	60	
Haemophilia A plasma +Normal human serum	>60	>60	55	22	20	12	18	18		
Horse 1. Plasma (control)	>60	>60	>60	>60	53	41	33	23	24	24
Horse 1. Plasma+ Normal human plasma	>60	60	20	15	12	11	13	14		
Horse 1. Plasma+ Haemophilia B plasma	>60	>60	>60	50	40	25	21	18		
Horse 1. Plasma+ Normal serum	—	—	16	9	11	—	11			
Horse 1. Plasma+ Haemophilia B serum	—	—	56	20	15	13	13	12	12	
Horse 2. Plasma (control)	>60	>60	>60	>60	37	23	15	15		
Horse 2. Plasma+ Horse 2 serum	—	—	>60	28	20	16	11·5	9·5	10	
Horse 2. Plasma+ Haemophilia B serum	—	—	>60	46	32	18	14	12	11	

Haemophilia A indicates deficiency of antihaemophilic globulin (A.H.G.)
Haemophilia B indicates deficiency of the Christmas factor (P.T.C.)

Thrombin Generation Test

The results are shown in Table 2. A significant improvement was found after the addition of normal human plasma, human haemophilia A plasma (deficient in A.H.G.), normal human serum, human haemophilia B serum (deficient in Christmas factor) and the corresponding horse serum, although the degree of correction varied from animal to animal. Furthermore, the activity of the plasma increased spontaneously on standing over a period of 1 to 2 hours. After storage at —25°C. for 24 hours, the animal plasmas were as active as the human plasmas. Unlike the thromboplastin generation test, storage markedly affected the results of the thrombin generation test. The animal's own serum improved the thrombin activity of its plasma, and normal serum improved the activity of known anti-haemophilic globulin deficient plasma.

Platelet Thromboplastic Activity

This was assayed in combination with normal human $Al(OH)_3$ plasma and with both normal human serum and horse serum. The platelet concentration in the reaction mixture was between 400,000 and 550,000 per cu.mm. The activity with horse serum was significantly better than that with human serum (Table 3). Under these conditions human platelets in combination with human serum are usually more active and produce minimum substrate clotting times of 10 to 11 seconds.

TABLE 3

THE THROMBOPLASTIC ACTIVITY OF HORSE PLATELETS

Platelets	*Substrate clotting time (sec.) Incubation time (min.)*								*Concentration of platelets in reaction mixture per cu.mm.*
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	
Horse 1+Human serum	63	39	25	20	24				430,000
Horse 1+Horse serum	55	33	15	15	15				
Horse 2+Human serum	70	47	26	19	18	18			570,000
Horse 2+Horse serum	56	35	16	15	14	14			
Horse 3+Human serum	70	57	37	25	24	27			410,000
Horse 3+Horse serum	71	46	23	16	16	16			

Normal human $Al(OH)_3$ plasma used throughout

DISCUSSION

This investigation confirms the previously reported observation of Bell *et. al.*, (1955) that horse plasma has less anti-haemophilic globulin activity than human plasma. The prolonged one-stage

plasma prothrombin time which they found in both horses and ponies was also confirmed when human brain thromboplastin was used; however, when horse brain thromboplastin was used the prothrombin times were shorter than those found with human plasma. Therefore, when the homologous thromboplastin was used there did not appear to be any defect of the prothrombin complex or of the thrombin-fibrinogen reaction in the horse. When horse thromboplastin was used in combination with human plasma, the prothrombin times of the latter were prolonged in a manner similar to that found for the human brain-horse plasma combination.

While horse and human brain thromboplastin could not substitute for one another in the one-stage plasma prothrombin tests, they were equally active in determining the one-stage serum prothrombin times. This observation is in keeping with the finding of Mann and Hurn (1952) that the species differences of various thromboplastins disappeared when they were exposed to the homologous serum. On the other hand, the lipid fractions of human and horse brain could substitute equally well for platelets in the thromboplastin generation test. With respect to the activity of these two lipids, there did not appear to be any species difference. Since it is believed that the thromboplastic material used in the one-stage plasma prothrombin test is a lipo-protein complex (Cohen and Chargaff, 1940), the species difference under these conditions is probably associated with differences in the protein component of this complex.

The results of the thrombin generation tests are difficult to evaluate. Improvement of the plasma thrombic activity with various sera, including that of the animal itself, might suggest that some non-specific accelerator material was being supplied. The correcting effect of haemophilia A plasma appeared to be variable. Spontaneous improvement in the plasmas on standing made the interpretation of results even more difficult at times. Our experience with this test makes us feel that its use in the investigation of a coagulation defect is not necessarily very helpful, and indeed, may be confusing. Using this test, Sjølin (1956) has claimed that the decreased thromboplastic activity of horse blood is the result of a Christmas factor deficiency and not of anti-haemophilic globulin lack. We were unable to obtain such clear-cut evidence using the thrombin generation test. On the other hand, with the thromboplastin generation test the evidence points clearly to a decrease in anti-haemophilic globulin activity.

The thromboplastic activity of horse platelets appeared to be less than that of human platelets; their activity was better with horse serum (although still relatively less) than with human serum. As the assay method used human plasma, as a source of A.H.G., at least some of the activity decrease might be the result of some lipoprotein species differences in platelet composition rather than an actual decrease in the true platelet thromboplastic factor which is

presumably a pure lipid. This aspect of the problem, however, requires further work before a firm conclusion can be reached.

The activity of horse plasma with Russell's Viper Venom approximated that of human plasma. In this respect, the snake venom thromboplastin, unlike the human and horse brain thromboplastin, showed no species difference. This provided further support for the contention that there is no difference in the prothrombin complex activity and in the thrombin-fibrinogen reaction in the two plasmas. Furthermore, the fact that substrate horse plasma was as good as substrate human plasma in the thromboplastin generation test lent additional support to this interpretation.

The question as to how far it is valid to interpret the coagulation findings in horse blood in terms of human values is of some importance in deciding on their implications. The apparent thromboplastic deficiency in the horse may not have the same biological significance for the horse as a similar finding would have for man. Clearly the horse has none of the clinical disturbances found in human haemophiliacs. The thromboplastin activity level as determined with a particular technique may well be "normal" for the horse.

CONCLUSIONS

Studies were made on some aspects of the blood coagulation mechanism of normal horses and ponies, relative to human blood.

Horse and pony blood showed a deficiency of anti-haemophilic globulin activity. The Christmas factor activity appeared to be unimpaired.

The activity of the prothrombin, factor V, factor VII and the thrombin-fibrinogen reaction in horse plasma was at least equal to that of human plasma.

Horse platelets were thromboplastically more active when combined with horse serum than with normal human serum, but appear less active than human platelets. Ponies had higher platelet counts than horses.

Species differences were found in the activity of the brain thromboplastins (lipoprotein) but not in that of the brain lipids. Human and horse plasmas were equally active in the presence of snake venom thromboplastin.

The possible significance of the findings is discussed with reference to their biological implications.

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FURTHER EXPERIMENTS ON THE EFFECT OF TICK-BORNE FEVER INFECTION ON THE SUSCEPTIBILITY OF LAMBS TO STAPHYLOCOCCI

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INTRODUCTION

In earlier experiments (Foggie, 1956) it was shown that the intravenous inoculation of numbers of staphylococci insufficient to produce pathological changes in normal lambs would produce pyaemia in lambs inoculated during the neutropaenia following an attack of tick-borne fever. The following experiments were carried out to determine whether subcutaneous inoculation of staphylococci in neutropaenic animals would also produce pyaemia, a term used to indicate staphylococcal abscess formation anywhere in the body except at the inoculation site. The experimental animals used were mice rendered neutropaenic by intravenous injection of nitrogen mustard, and lambs in the neutropaenic phase of tick-borne fever. The effect on neutropaenic lambs of staphylococci given intradermally was also tested.

METHODS

Animals. Mice of both sexes between 5 and 8 weeks old were used in these experiments. The lambs used were the progeny of a flock of Blackface ewes maintained at the Institute throughout gestation. As in previous experiments (Foggie, 1956) pairs of lambs of the same age were selected; one lamb in each pair was infected with tick-borne fever by the intravenous inoculation of a pooled sample of blood from 3 donor sheep. Periodic white cell counts were carried out and when a neutropaenia appeared in the tick-borne fever infected lamb, both lambs were inoculated with staphylococci.

Staphylococcus. Strain 1468 was maintained in Worth's medium at room temperature. The inoculum was prepared either from a saline suspension of an overnight agar culture standardised to No. 8 of Brown's opacity tubes which contained approximately 3×10^9 organisms per ml. or from an overnight broth culture which contained approximately 75×10^6 organisms per ml.

RESULTS

Mice

Comparison of Intravenous and Subcutaneous Routes

In a series of experiments it was found that the injection of mice with staphylococci (strain 1468) by the intravenous and subcutaneous routes produces two quite distinct types of lesion. By the intravenous route, provided the mouse does not die within

48 hours of inoculation, the typical picture is abscess formation in the internal organs, joints and/or muscles, the kidneys being most commonly involved. Subcutaneous inoculation results in a local lesion characterised by necrosis of the skin which is under-run with pus and eventually sloughs, but secondary abscess formation is extremely rare although following injection of large doses generalisation of the infection as determined by cultivation of staphylococci from the internal organs is common. The number of staphylococci given subcutaneously which will produce generalised infection is about 40 times that which will produce generalisation or pyaemia when given intravenously.

In a typical experiment 6 groups of 20 mice were inoculated subcutaneously over the sacrum and 5 groups of 20 mice were inoculated intravenously with 0·1 ml. of dilutions of a suspension containing 3×10^9 staphylococci per ml. (Table 1). In addition to the mice that died, one mouse from each group was killed each day and examined for lesions; cultures were made from the liver, lung, heart, spleen and kidneys.

The majority of mice inoculated intravenously with the lower dilutions of staphylococci developed pyaemia but none of the mice inoculated subcutaneously showed secondary abscess formation.

TABLE 1

INOCULATION IN GROUPS OF 20 NORMAL MICE

Dilution of staphylococcal suspension	*No. of mice showing pyaemic lesions*	*No. of mice with generalised staphylococcal infection*	*No. of mice culturally negative except for local lesions*	*No. of mice showing local lesions*
A. Subcutaneous inoculation				
1/1	0	16	4	20
1/2	0	14	6	20
1/5	0	8	12	19
1/10	0	3	17	12
1/20	0	3	17	10
1/50	0	0	20	8
B. Intravenous inoculation				
1/20	14	4	2	
1/50	12	2	6	
1/100	12	1	7	
1/200	6	5	9	
1/500	1	2	17	

The staphylococcal suspension was standardised to No. 8 of Brown's scale opacity tubes.

Intravenous Inoculation in Neutropaenic and Normal Mice

Mustine hydrochloride B.P.C. (nitrogen mustard) has been used to produce neutropaenia in rabbits (Humphrey, 1955). A preliminary experiment on mice showed that a single intravenous injection of 2·5 mg. nitrogen mustard/kg. body wt. produced a consistent

neutropaenia on the fourth day after injection and that the majority of the mice recovered.

To compare the effect of the intravenous inoculation of staphylococci in neutropaenic and normal mice groups were inoculated intravenously with .2·5 mg./kg. body wt. of nitrogen mustard. Comparable groups of mice were left untreated. Four days later, treated and untreated mice were inoculated intravenously with 0·1 ml. of a dilution of an overnight broth culture of staphylococci. To determine the lethal effect of nitrogen mustard alone, a group of 19 mice treated with nitrogen mustard was left uninoculated with staphylococci; only one died. On the fourth day after the inoculation of nitrogen mustard the mean neutrophil count per cu.mm. of blood from 29 of the nitrogen mustard-treated mice was 354 ± 431 and on 20 of the untreated mice 2969 ± 1919, showing that the nitrogen mustard inoculation had produced a highly significant reduction in neutrophils.

Following the inoculation of staphylococci, deaths were recorded for 8 days, after which the surviving mice were killed. Cultures were made from the liver, lungs, heart, spleen, both kidneys and any macroscopic lesion.

The effect of the intravenous inoculation of 10^{-2} and 10^{-3} dilutions of staphylococcal culture is shown in Table 2. The presence of neutropaenia lowered the resistance of the mice to the staphylococci. When challenged with a 10^{-2} dilution of culture there was a highly significant difference between the death rates of neutropaenic and normal mice ($\chi^2 = 15·22$) but with this dose the majority of the normal mice as well as all the surviving neutropaenic mice developed pyaemia. Following the inoculation of 10^{-3} dilution of culture one neutropaenic and one normal mouse died with internal abscesses. A comparison of the two groups shows that abscess formation was significantly more frequent in the neutropaenic mice than in the normal mice ($\chi^2 = 6·876$).

TABLE 2

INTRAVENOUS INOCULATION OF DILUTIONS OF CULTURE IN NEUTROPAENIC AND NORMAL MICE

Mice	*Dead*	*Pyaemic*	*Healthy*
10^{-2} dilution			
Neutropaenic	13	5	0
Normal	1	12	3
10^{-3} dilution			
Neutropaenic	1	9	8
Normal	1	1	14

Subcutaneous Inoculation in Neutropaenic and Normal Mice

Twenty neutropaenic and 20 normal mice were inoculated subcutaneously over the sacrum with 0·1 ml. of a suspension containing

approximately 12 × 10^7 staphylococci per ml. Nine of the neutropaenic mice and 3 normal mice died and the remainder were killed 8 days after the inoculation. Although staphylococci were isolated in culture from the organs of 9 of the neutropaenic mice and 6 of the normal mice, apart from the inoculation lesion, there was no evidence of abscess formation in either group.

The most noticeable difference between the surviving mice in the two groups was in the size of the local lesions, the average diameter in the mice treated with nitrogen mustard being nearly twice that of untreated mice. Smears from the inoculation site of the treated mice that died on the second day after inoculation showed a nearly complete absence of neutrophils, whereas in an untreated mouse that died on the day following inoculation a marked neutrophilic infiltration was present.

These experiments show that subcutaneous inoculation is not followed by secondary abscess formation in either neutropaenic or normal mice.

Lambs

Neutrophil Counts

White cell counts on 35 normal lambs between the ages of 10 and 45 days gave a mean of 1357 ± 814 neutrophils per cu.mm. of blood. The mean of the lowest recorded neutrophil counts in 19 lambs following experimental tick-borne fever infection was 220 ± 129 cells per cu.mm. The difference between the means is significant ($t = 2{\cdot}028$, $P < {\cdot}05$). In the staphylococcal inoculation experiments a count of less than 500 was considered as indicative of a neutropaenia.

Intravenous Inoculation

A previous experiment (Foggie, 1956) indicated that the neutropaenia following an attack of tick-borne fever reduced the resistance of lambs to the intravenous inoculation of staphylococci. The experiment was repeated and confirmed (Table 3).

The neutropaenic lambs inoculated with 10^{-2}, 10^{-3} and 10^{-4} dilutions of an overnight broth culture of staphylococci all developed pyaemia. Those inoculated with 10^{-5} and 10^{-6} dilutions showed no symptoms. The control lamb which was inoculated with 10^{-2} had a temperature of over 105°F. on the sixth and seventh days after inoculation. It was killed and examined on the tenth day; small haemorrhages were found in the lungs and kidneys. Cultures from the liver, lungs, heart blood, spleen and kidneys were, however, all negative indicating that the infection had been overcome. The other control lambs remained healthy.

Subcutaneous Inoculation

Four pairs of lambs, one lamb of each pair being neutropaenic and the other normal, were inoculated subcutaneously behind the shoulder with 1 ml. of broth culture or 10^{-1}, 10^{-2} and 10^{-3} dilutions

TABLE 3

INTRAVENOUS INOCULATION IN LAMBS DURING NEUTROPAENIA AND IN NORMAL LAMBS

	Tick-borne fever infected lambs			*Normal lambs*		
Staphylococcal inoculum 1 ml. of broth dilutions	*Neutrophil count at inoculation (cells/cu.mm.)*	*Disposal*	*Clinical or post-mortem findings*	*Neutrophil count at inoculation (cells/cu.mm.)*	*Disposal*	*Clinical or post-mortem findings*
10^{-2}	196	Died	Pyaemia	1301	Killed	Miliary haemorrhages negative on culture
10^{-3}	478	Killed	Pyaemia	687 (114)	Survived	Normal
10^{-4}	180	Killed	Pyaemia	3150	Survived	Normal
10^{-5}	217	Survived	Normal	422 (592)	Survived	Normal
10^{-6}	72	Survived	Normal	553 (276)	Survived	Normal

Figure in brackets = eosinophil count.

of the culture. None of the lambs developed pyaemia. One normal lamb inoculated with undiluted culture and one neutropaenic lamb inoculated with a 10^{-3} dilution developed cellulitis and died within 3 days of the inoculation. In the remaining lambs the local lesion was more severe in the neutropaenic than in the normal lambs.

Intradermal Inoculation

This experiment was carried out in the same way as the previous one except that doses of 0·1 ml. of undiluted culture or 10^{-1} and 10^{-2} dilutions thereof were inoculated intradermally. The reactions were entirely local and were larger in the neutropaenic than in the normal lambs.

Repeated Subcutaneous Inoculation

As it was possible that the critical stage in the neutropaenia was being missed when a single subcutaneous inoculation of staphylococci was given, three lambs were inoculated with tick-borne fever and as soon as infection was diagnosed by elevation of temperature and the demonstration of tick-borne fever inclusion bodies in the neutrophils, daily subcutaneous inoculations of 0·1 ml. of a 10^{-3} dilution of culture were made, a different site for inoculation being selected each day. Neutrophil counts were made every second day.

One of the lambs developed an abscess between the digits of the right fore-foot on the day after it had received the second injection, and the inoculations were stopped. Bacteriological examination of pus from this abscess however yielded a pure culture of *Corynebacterium pyogenes*. Another lamb developed a staphylococcal cellulitis of the right hind leg after receiving 8 daily inoculations. The sixth inoculation was given in the right thigh when the neutrophil count was 37 cells per cu.mm. and it is probable that this inoculation was responsible for the cellulitis. The two subsequent inoculations, given in the left thigh and right axilla, produced no visible lesions. The third lamb was given inoculations on 10 successive days during which period the neutrophil count dropped to 326 cells per cu.mm. and returned to a level of 1350 cells per c.mm., but no local or general lesions developed. Three normal lambs also received 10 daily injections without showing either local or general reactions.

These experiments appear to indicate that even in the neutropaenic animal pyaemia can only be produced by the direct introduction of staphylococci into the blood stream.

Bacteriostatic Action of Normal and Tick-borne Fever Infected Blood

An in vitro experiment was carried out to see if it could be demonstrated that the tick-borne fever infection upset the clearance mechanism of the blood for staphylococci. To avoid interference from the coagulase reaction, defibrinated blood was used throughout the experiment, and in order to reduce damage to the white cells during defibrination a coil of wire was substituted for beads in the collecting

bottle. Volumes of 4 ml. of defibrinated blood were added to tubes containing 1 ml. of serial dilutions of an overnight broth culture. After sampling, the tubes were closed with sterile rubber stoppers and incubated at 37°C. on a rotator which inverted each tube 5 times per minute. Samples were removed from the tubes after various periods of incubation by pipettes calibrated to deliver a drop of 0·05 ml. volume and 4 drops of blood were allowed to fall on to a sheep-blood agar plate. Each drop was spread over a quarter of the plate, which was then incubated. Counts of staphylococcal colonies were made the following morning.

Normal sheep blood had the effect of prolonging the lag phase in the growth and reducing the colony count after 2, 3 and 4 hours incubation. By the sixth hour of incubation growth was usually well established. Occasionally the count from the 6 hour sample remained low and in one or two tests complete sterilisation of the higher dilutions in normal blood occurred. Tests in which serum with a high α antihaemolytic titre and serum with a high complement fixing titre to staphylococcal cells were substituted for normal blood showed that serum alone had no bacteriostatic action. In each series of tests on normal blood and tick-borne fever blood, a test with normal serum was included as a control.

TABLE 4

BACTERIOSTATIC ACTION OF SERUM, NORMAL BLOOD AND TICK-BORNE FEVER INFECTED BLOOD

Date	*Serum*	*Normal lamb blood*		*Tick-borne fever infected lamb* blood*	
	Index	*Neutrophil count*	*Index*	*Neutrophil count*	*Index*
1/6/56	0·75	966 (80)	10·20	813 (187)	5·25
5/6/56	1·02	488 (81)	2·50	1300	1·83
8/6/56	0·91	1687	13·56	90 (20)	0·49
12/6/56	0·56	866 (79)	1·88	578	1·55
15/6/56	0·70	625	11·13	306	1·23
20/6/56	0·83	861	4·75	86 (172)	1·00
26/6/56	0·77	571 (163)	5·46	660 (330)	4·21

$$\text{Index} = \frac{\text{Colony count before incubation}}{\text{Colony count after 3 hours incubation}}$$

Figure in brackets = eosinophil count.

* Lamb inoculated with tick-borne fever on day O. Temperature reaction from 3rd to 11th days.

A six week old lamb was inoculated with tick-borne fever blood. The temperature rose on the third day and returned to normal on the eleventh. Starting on the first day blood samples from this lamb and from a normal lamb of the same age were tested for bacteriostatic action against staphylococci at intervals for 26 days. At each sampling a white cell count was made on the blood of both lambs.

The interpretation of the results of the experiment is based on the plate counts from tubes made with 10^{-5} dilutions of culture. The degree of bacteriostasis is expressed as an index which is arrived at by dividing the colony count before incubation by the colony count after 3 hours incubation of the blood staphylococcal mixture. An index of less than one indicated a larger colony count at 3 hours than in the initial sample, while an index of more than one showed reduction in colony numbers after incubation (Table 4). In tests of serum there was a fairly constant increase of about 20 per cent. in the colony counts between the initial samples and the 3 hour samples. The indices for the normal lamb's blood were very variable, but the smallest showed approximately a 2 fold reduction and the largest a 13 fold reduction in colony counts. There was little correlation between the indices and the neutrophil count. In the lamb affected with tick-borne fever however the index fell from a preinfection figure of 5·25 to 0·49, 5 days after the beginning of the temperature reaction and remained below 2 for a further 12 days, a period which conformed in general with the duration of the neutropaenia.

DISCUSSION

These experiments on mice and lambs showed that the subcutaneous or intradermal inoculation of the normal animal resulted in a local lesion which varied in size with the numbers of staphylococci injected and sometimes led to extensive cellulitis. Staphylococci were occasionally recovered in culture from the spleen, kidney, liver, lungs or heart, but death when it occurred appeared to be due to a toxaemia, originating from the local lesion. Secondary abscess formation was not detected. When staphylococci were injected subcutaneously in a neutropaenic animal they multiplied apparently unhindered at the injection site until the return of neutrophils limited the spread of the lesion. The resulting lesion was similar to that produced by a much larger inoculum in a normal animal but pyaemia did not occur.

The intravenous injection of sufficiently large numbers of staphylococci in both lambs (McDiarmid, 1948; Foggie, 1948) and mice led to the appearance of single or multiple abscesses in various parts of the body. In the neutropaenic animal the pathological picture was the same, but it could be produced by much smaller numbers of staphylococci, a finding which suggests that it is the organisms which escape phagocytosis in the circulating blood that are responsible for the production of abscesses.

These experiments confirm the findings of Taylor, Holman and Gordon (1941) who failed to produce pyaemia by the subcutaneous inoculation of staphylococci in lambs suffering from an attack of tick-borne fever, but they throw no further light on the method by which the staphylococcal infection reaches the blood stream in tick pyaemia. They do, however, suggest that the pyaemia is not caused by invasion from an already existing superficial lesion, a point which is supported by examinations of numerous field cases of the disease in which no macroscopic cutaneous or subcutaneous lesion could be detected.

CONCLUSIONS

The previous finding that lambs in the neutropaenic phase of tick-borne fever are more susceptible than normal lambs to pyaemia following the intravenous inoculation of staphylococci, was confirmed. A similar increased susceptibility to the intravenous inoculation of staphylococci was found in mice rendered neutropaenic by the injection of nitrogen mustard.

The subcutaneous and intradermal inoculation of staphylococci in neutropaenic lambs and mice failed to produce pyaemia, but the resulting local lesions were larger than those produced by similar numbers of micro-organisms in normal animals. Although no secondary abscesses were produced, staphylococci could occasionally be cultivated from the internal organs of both neutropaenic and normal animals inoculated subcutaneously.

An *in vitro* bacteriostatic test on the blood of a normal lamb and a lamb infected with tick-borne fever indicated that during the tick-borne fever neutropaenia there was a reduction in bacteriostasis for staphylococci.

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OSTEOPOROSIS IN LAMBS IN A LEAD MINING AREA

I. A Study of the Naturally Occurring Disease

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INTRODUCTION

This communication describes an osteoporotic condition of young lambs, characterised by a stiff gait, lameness or posterior paralysis and unthriftiness, which has occurred regularly for more than 50 years in a former lead mining area in Southern Scotland but has not been previously recorded. So far it has been identified pathologically on seven farms in the district and the reports of shepherds suggest that it occurs on several more.

The affected area is at an altitude of 1,000 to 1,500 feet and scattered over the hillsides are numerous waste heaps from the mines which were closed for economic reasons about 20 years ago. The ruins of two smelting mills with their ore washing beds are situated on the banks of streams which, when in flood, still carry away waste material and deposit it further down the valleys. Most of the grazing is unfenced and the sheep have access to the mine workings and the valleys and hillsides contaminated by the smelting mills which they seem to prefer to other pastures. Shepherds and flock-masters have associated the disease with these contaminated areas for many years and accordingly herd the sheep away from them as much as possible. In general the incidence of the disease appears to be related to the efficiency with which this can be carried out. It is also recognised locally that if hens and ducks are allowed free range over these areas they too pine and die.

MATERIALS AND METHODS

Our study of this condition has been carried out on the lamb crops of 3 successive years, i.e. 1953, 1954 and 1955, on 7 farms in the area. In 1953 and 1954 a preliminary investigation was made of 12 cases and since this indicated the presence of an osteodystrophic disease the cases obtained in 1955 were examined in greater detail, particular attention being paid to the skeleton. In the previous years it had been found that one farm in particular had a high incidence of the disease, and in 1955 efforts were made to examine all available cases from this farm (Farm B). In fact 14 out of the 20 lambs examined post-mortem in that year came from this farm. It was also visited at marking-time (June 20th) and every lamb was examined as thoroughly as possible for evidence of skeletal abnormalities. Three lambs which appeared clinically normal at this

examination were selected for post-mortem examination. Sixteen of the 20 lambs examined in 1955 were males. Nineteen were pure Blackface lambs, while the other was a Blackface cross.

Pathological investigations. In 1955 blood samples were taken from each lamb for haematological and biochemical examination. The lambs were then slaughtered, and all organs, bones and joints were examined. Material for histological examination was taken from all the body organs and from the limb bones on the left side, the skull, thoracic, lumbar and coccygeal vertebrae, the ribs, costochondral junctions and pelvis, and from all other bones showing macroscopic abnormality. All tissues were fixed in 10 per cent formol saline. Bone blocks were decalcified in 5 per cent. nitric acid by the method described by Clayden (1952).

Biochemical investigations. Analyses were carried out on blood and tissue samples from the 20 cases obtained in 1955 and from 8 additional cases examined during the two previous seasons. Pathological examination showed that they were all affected by osteoporosis. For comparative purposes parallel investigations were carried out on normal lambs of the same breed and age group which were reared at Moredun Institute. Blood serum was analysed for calcium by the Clark and Collip modification of the Kramer-Tisdall method (Clark and Collip, 1925) and for magnesium by the method of Denis (1922) modified according to Hawk (1947). Total and inorganic phosphate were determined in a trichloracetic acid extract of whole blood by the method of Fiske and Subba Row (1925) and haemoglobin by a modification of the procedure published by Cohen and Smith (1919).

The ash, calcium and phosphorus content of the entire fourth rib taken from the right side was determined by a modification of the procedure adopted by Sobel, Rochenmacher and Kramer (1944). Lead was determined in whole blood, the ventral lobe of the liver, the right kidney, the entire fifth rib from the right side, and in lumbar vertebrae with dithizone using the reversion technique (Irving and Butler, 1953). This method was also used for the analysis of pasture and soil samples collected from the affected area in 1954 while the disease was occurring.

Since zinc ores occur in workable quantities in the district and an excess of this metal had been shown to produce unthriftiness and lameness in pigs (Grimmett, McIntosh, Wall and Hopkirk, 1937; Sampson, Graham and Hester, 1942) zinc estimations were carried out on some of the tissue samples. The 9 liver samples which were analysed were from the most severely affected cases. An absorptiometric dithizone procedure was used (Butler, 1952) and strict precautions were taken to minimise contamination of the samples by adventitious traces of zinc; for instance all-steel needles were used for the collection of blood samples (Butler and Newman, 1956) and the use of face powder was prohibited in the analytical laboratory (Butler and Field, 1956).

RESULTS

Clinical Features

The principal feature of the disease is an excessive fragility of the bones due to osteoporosis. This is responsible for a variety of locomotor disturbances ranging from a stiff gait, which may only become apparent after the lambs have been driven for some distance, to complete posterior paralysis. These symptoms appear when the

lambs are between 3 and 12 weeks old, that is from the middle of May, when they have begun to graze, until the beginning of July, and never appear in adult sheep.

Lambs with a stiff gait walk slowly, taking short steps with minimum flexion of the joints and on a hard flat surface they can be seen to walk on their toes, the heels seldom touching the ground. This stiffness may disappear after about 4 weeks. More severely affected cases show definite weakness or inco-ordination of the hind limbs, particularly when they are made to turn quickly. Posterior paralysis usually occurs quite suddenly in apparently normal lambs when they have been startled e.g. by the sheep dog, or while being restrained for castration and docking, but in a few cases it results from a progression of the stiffness or inco-ordination. The loss of motor power is variable but is often complete and the lambs are only able to move by using their forelegs, which appear unaffected, dragging the hind limbs usually either straight behind or to one side of the body (Fig. 1). These cases never recover and soon die from secondary causes such as starvation, drowning or pneumonia.

Lameness occurs less frequently and is due to deformation or fracture of the fore or hind limbs, which may produce a bowed or a knock-kneed appearance when the fore limbs are affected (Figs. 2 and 3). The bones of lambs in affected flocks fracture readily from trivial causes, both under natural conditions and when normal restraint is applied e.g. at marking time in June.

Another manifestation of osteoporosis is the characteristic lesion of "cappi" or "double scaup" described by Bosworth and Stewart (1933) which occurs frequently in lambs showing locomotor disability, and also in lambs and hoggs in the same flocks which otherwise appear normal. The plate of bone overlying the frontal sinus becomes abnormally thin and yields to pressure on palpation.

The clinical features of the 20 lambs examined in 1955 were briefly as follows:—

Posterior paralysis	5
Inco-ordination of hind limbs	2
Lameness and deformities of limb bones	3
Lameness and thinning of the frontal bone	2
Stiff or lame, with swollen limb joints ..	3
Stiff	2
Clinically normal	3
Total	20

The incidence of cases of locomotor disability in the lamb crop varies between about 5 and 18 per cent., but 3 clinically normal lambs selected at random from one severely affected flock showed extensive manifestations of osteoporosis on post-mortem examination, indicating that the true incidence of the disease is very much higher. However the main cause of financial loss to the farmers is the general

unthriftiness of the lambs in these flocks which usually persists until the autumn.

Anatomical Findings

The majority of the 32 lambs examined were in poor condition, especially those showing obvious symptoms of paralysis or lameness, and several were undersized.

Skeletal lesions. The most striking abnormality affecting half of these lambs was a lesion involving one or more of the lumbar vertebrae, which caused constriction of the spinal cord and hence the posterior paralysis in the most severely affected lambs and the stiffness or inco-ordination of the hind limbs in those in which the lesions were less marked. In each case removal of the arches of the lumbar vertebrae and the spinal cord exposed a rounded or sometimes sharp bony prominence arising from the dorsal surface of the body of one or more of the vertebrae, projecting into the spinal canal and compressing the spinal cord against the arch above.

A vertical section made by sawing down the midline of the lumbar region of the vertebral column showed the bodies of affected vertebrae to be compressed in an anteroposterior direction. As a result of this compression the dorsal surface of the body had become forced upwards into the spinal canal, forming the prominence already mentioned (Figs. 4 and 5). In the more severe cases the trauma to the spinal cord had resulted in subdural haemorrhage. This lesion was found in all lumbar vertebrae from the second to the seventh inclusive. It usually occurred in only one, but in one lamb two vertebrae, the third and the sixth, were affected (Fig. 4) and in several other cases incipient lesions were observed. In 2 cases there was in addition a complete transverse fracture through the body of a lumbar vertebra, one of which occurred through the lesion. In each case the fracture had occurred close to the epiphyseal cartilage.

The midline section of the lumbar region frequently revealed herniation of the nucleus pulposus of the intervertebral discs into the bodies of the vertebrae (Fig. 4 and 5). In some cases the nucleus had herniated only through the end-plate of cartilage into the epiphysis, while in others it had penetrated through both the end-plate and the epiphyseal cartilage into the spongiosa of the vertebral body. A further abnormality was that the cancellous bone in the bodies of the lumbar vertebrae was deficient in amount and had a more delicate structure than normal. The marrow had a dark red hyperplastic appearance in many cases. This lesion with its dramatic consequences was the only skeletal abnormality present in 3 of the 20 cases examined in 1955, but in 14 of the remainder there was abundant evidence of a generalised osteodystrophy. Many fractures, both recent and healed, were found in those bones of the lamb's skeleton most exposed to stress and strain, e.g. the ribs, metacarpal and metatarsal bones, humerus, femur, pelvis and tuber calcis.

Rib fractures occurred in the middle third of the shaft and were often multiple, involving up to 9 ribs on one side of the chest. The fractures of long bones showed a tendency to occur at the extremity of the shaft close to the epiphyseal cartilage. Healed fractures of this type affecting the metacarpal and metatarsal bones produced deformities and curvature of the limbs (Figs. 2 and 3). Fractures were also found within joint cavities, varying from fissures on the articular surfaces of the bones to complete separation of fragments e.g. of the lateral epicondyle of the humerus.

Apart from gross fractures, other evidence of osteodystrophic disease was found. Thinning of the frontal bone of the skull was apparent in 9 lambs out of the 20 examined in 1955, this defect being so marked in one case that four holes were present in the bone near the midline, the largest being 0·75 cm. in diameter. In two cases the cancellous bone in the head of the humerus had been insufficiently strong to withstand the weight applied to it and had collapsed, causing the articular surface to become flattened and set at right angles to the shaft.

Common sites of minor deformity were the distal extremities of the metacarpal and metatarsal bones, where the articular ridges of the condyles were displaced to one side. Intact long bones of affected lambs when dissected out were more delicate and slender in appearance than those from normal lambs of the same age and breed. This fact became even more apparent when these bones were sawn in half longitudinally. The marrow, which was dark red, was found to extend almost to the epiphyseal discs as a result of the deficiency of cancellous bone in the metaphyses, which was particularly marked immediately below the centre of the epiphyseal disc (Fig. 6). The cortical bone of the shaft in the metaphyseal zone was also noticeably thinner than in the control animals. These changes were most readily seen in the proximal extremity of the tibia. Post-mortem examination of the 3 lambs selected as being clinically normal showed lesions of a similar nature in the ribs, skull and long bones.

The 3 lambs with swollen limb joints were found to be suffering from streptococcal joint-ill. Apart from the lesions of suppurative polyarthritis no other gross abnormality was observed in the skeletons of these lambs.

Visceral lesions. In the 20 lambs examined in 1955 the only significant findings in the viscera were in the kidney and liver. Three lambs showed hydronephrosis. Two of these had spinal lesions which had probably interfered with the micturition reflex. The kidneys were enlarged and pale fawn in colour; the capsule stripped easily and the surface below showed a diffuse faint yellow mottling. The pelvis of each kidney contained a considerable quantity of urine. The kidneys of other lambs, while of normal size, showed similar mottling of the external surface.

The livers of the majority of the 20 lambs showed centrilobular degeneration.

Histopathology

Bone. The skeletal lesions were the most important feature of this condition and were of a similar character in all 20 lambs examined, the only variation being one of degree. They were those of a generalised osteoporosis—what bone was present was of normal structure, but there was a deficiency, usually marked, in the amount of it.

The terminology used to describe bone diseases of nutritional origin has often been loosely applied, and this has been undoubtedly responsible for much confusion and misinterpretation. Here the term osteoporosis refers to the condition defined by Albright (1947) as the diminution of bony mass resulting from decreased production of osteoid tissue (bone matrix), and associated with normal calcification of that osteoid tissue which is laid down. Osteoporosis was present in all the bones of the skeleton which were examined, from the skull to the coccygeal vertebrae, but affected most severely the ribs and costo-chondral junctions, the vertebrae, the frontal, metacarpal and metatarsal bones and the tibia.

The principal histological features may be summarised as follows:— The epiphyseal cartilages of the long bones were reduced to approximately half their normal thickness due to a decrease in the depth of the proliferating cell zones. The cartilage columns, however, remained perfectly regular. Inhibition of osteoblastic acitvity had caused a reduction in the amount of osteoid tissue deposited on the spicules of calcified cartilage matrix. As a result, the trabeculae in the metaphyseal region were extremely slender and in the most severely affected lambs were also greatly reduced in number (Figs. 7 and 8). This deficiency of trabeculae was most apparent below the central part of the epiphyseal disc, while an attempt at the maintenance of more normal structure was seen at its periphery.

Near the junction of shaft with epiphyseal cartilage cortical bone also was very deficient, the combined effect of these changes being to render the bones liable to transverse fractures both macroscopic and microscopic in this weakened metaphyseal zone. The borders of such fractures were commonly delineated by intensely eosinophilic material, and were often associated with small haemorrhages. Cortical bone from the mid-shaft of such bones as the tibia and femur also showed porotic changes, with abnormally wide Haversian canals and persistent cartilage cores. The surface bounding the medullary cavity showed resorptive changes consisting of an irregular saw-toothed border associated with numbers of osteoclasts.

The cancellous bone of the epiphyses showed a similar delicate structure (Figs. 9 and 10) sometimes unable to withstand the normal stresses imposed on it.

The appearance of the osteoblasts was variable. In those cases showing most osteoporotic change they were much fewer than normal, and appeared as mere elongated, flattened, hyperchromatic nuclei with no stainable cytoplasm, closely applied to the bone

surface. In less severely affected bones they were plump with basophilic cytoplasm, and although present in greater numbers were still obviously incapable of laying down bone matrix in normal amount. The number of osteoclasts present also varied. In some cases they were few, while others showed numerous giant cells amongst the trabeculae. A number of these osteoclasts displayed the clumping of chromatin and vacuolation of cytoplasm described by Marsden and Wilson (1955).

The vertebrae showed changes exactly similar to those just described, viz. narrowing of the epiphyseal discs, very slender and widely spaced trabeculae and deficiency of cortical bone, and this general delicacy of structure accounted for the readiness with which the lumbar vertebrae collapsed under pressure and predisposed the nucleus pulposus to herniation as previously described (Fig. 5).

The frontal bone of the skull was a site which appeared particularly prone to osteoporotic change. In 9 of the 20 lambs examined the bone overlying the frontal sinus was extremely thin, varying from 0·25 to 0·50 mm. compared with the measurement obtained from normal lambs of similar age which is approximately 1 mm. The septum dividing the frontal sinuses in these lambs consisted largely of fibrous tissue and very little bone (Figs. 11 and 12). As a result of this it had in some cases collapsed, obliterating the sinus cavities. Sections of costochondral junctions and rib shafts showed changes typical of those described above (Figs. 13 and 14).

Healing of fractures. The apparent inhibition of osteoblastic activity described above also had a marked effect on the repair process in fractured bones, as would be expected. In those lambs showing most marked osteoporotic change the reaction at the site of fracture was very deficient. The osteogenic layer of the periosteum showed little increase in thickness, osteoblasts were few in number and only a narrow collar of cancellous bone formed the callus surrounding the ends of the bone fragments. The borders of the fracture were demarcated by margins of structureless material which was intensely eosinophilic. Further evidence of defective healing was provided by the fact that although the callus completely surrounded the ends of the fragments of bone and had formed a union between them, in many cases sections showed microscopic fissures traversing the callus, some extending almost completely across its diameter. Fissures of this type were particularly noticeable in the ribs. The numbers of osteoclasts associated with the resorption of dead bone were very variable. The gap between the fragments where the internal callus normally forms was largely filled by fibrous tissue. In lambs showing less marked osteoporosis there was a correspondingly more normal response at sites of fracture.

Kidney. In 5 of the 20 lambs examined, large numbers of acidophilic intranuclear inclusion bodies were seen in the cells lining the proximal convoluted tubules and the descending straight tubules, particularly in those situated in the inner zone of the cortex.

These bodies were similar to those described by Blackman (1936) in the kidneys and livers of children suffering from lead poisoning, and by Staples (1955) in the kidneys of dogs experimentally poisoned with lead. They were acid-fast in sections of kidney stained by the method described by Wachstein (1949), which however was inferior to haematoxylin and eosin for their demonstration, a fact which has previously been remarked upon by Marsden and Wilson (1955). In each case the tubular cells also showed fine cytoplasmic vacuolation.

The kidneys of the 3 lambs in which hydronephrosis was found showed the typical histological appearance of this condition with tubular atrophy, fibrous tissue replacement and tortuous collecting tubules. In 15 of the 17 lambs which showed no hydronephrosis there was evidence of a pathological change involving the glomerular tufts and the parietal layer of Bowman's capsule in the outer half of the cortex (Fig. 15). This process commenced in those cells of Bowman's capsule farthest from the vascular pole of the tuft, which altered from their normal simple squamous type to become cuboidal and in some cases columnar, and eventually involved the whole capsule. The capsular space was greatly distended, containing eosinophilic globules and amorphous material, and the junction with the proximal convoluted tubule was wide and visible in a much larger percentage of glomeruli than normal. Simultaneously pericapsular fibrous tissue was being laid down, involving in many cases the adjacent tubules. Initially this fibrosis caused an increase in the capsular space, probably as a result of contraction drawing the capsule centrifugally, and also produced an angular distortion of the parietal layer in many affected glomeruli. During this process the glomerular tuft atrophied until only a few capillary loops remained which were abnormally patent and devoid of glomerular epithelium. Finally the tuft vanished and the glomerulus was replaced by fibrous tissue. The glomeruli in the inner half of the cortex showed little abnormality. Vascular lesions beyond those already described were not evident. Patchy areas of interstitial fibrosis were present, but were confined mainly to the subcapsular zone of the cortex and between the tubules of the medullary rays. These areas remained slight in extent even in cases where periglomerular fibrosis was pronounced.

In 5 lambs these lesions were considerable in extent, affecting the majority of the glomeruli in the outer zone of the cortex. In the remaining 10 lambs the lesions were slight, affecting scattered glomeruli. The severity of the lesions was not related to the presence of inclusion bodies. Hartley (1953) has described similar changes in the kidneys of lambs in New Zealand suffering from a myopathy probably due to lack of Vitamin E.

Liver. Intranuclear inclusion bodies similar to those described in the cells of the kidney tubules were present in the hepatic cells of 3 of the 5 lambs which had shown inclusion bodies in the kidneys. They were much less numerous than in the kidney, a fact in accordance with the findings of Blackman (1936). These 3 lambs and 7

others showed moderate to advanced fatty change of the hepatic cells, most marked in the centrilobular zone.

Central nervous system. Sections of brain and spinal cord were stained by haematoxylin-eosin and Weigert-Pal methods. The only lesions observed were those of local trauma in the lumbar regions of the spinal cords of those cases showing vertebral collapse.

Radiological examination of bones. The tibias of 11 of the affected lambs were submitted to radiological examination. Varying degrees of osteoporosis were demonstrated, but there was no evidence of the 'lead line' or zone of increased density described by Park (1930) in children suffering from chronic lead poisoning.

Haematology. The haemoglobin values, the total red and white cell counts and the differential white cell counts showed no abnormalities. Punctate basophilia was present in the red blood cells of 4 lambs, and in 2 of these Howell-Jolly bodies were also seen.

Biochemical Results

Minerals in blood. The 3 cases with hydronephrosis showed high levels of calcium (31·8, 13·5 and 13·2 mg./100 ml. respectively), total phosphate (30·0, 20·5 and 20·0 mg./100 ml.) and inorganic phosphate (13·0, 11·5 and 9·95 mg./100 ml.), but the values for the remainder of the affected lambs did not differ significantly from those for the group of normal lambs, thereby confirming the conclusion drawn from the histological studies that this is an osteoporotic condition.

Minerals in bone. From the results summarised in Table 1 it can be seen that a conventional analysis of the dry fat-free ribs showed no abnormality in the affected lambs. This finding indicates that all the osteoid tissue which was present was calcified in the normal manner and provides further confirmation of the osteoporotic nature of the changes.

TABLE 1

MINERAL CONTENT OF RIBS OF 20 AFFECTED AND 8 NORMAL LAMBS
(% of dry, fat-free material)

Constituent	*Affected lambs*		*Normal lambs*	
	Range	*Mean*	*Range*	*Mean*
Ash	50·4–59·0	54·1	49·7–57·8	52·9
Calcium ..	17·1–22·8	19·3	16·3–21·6	19·4
Phosphorus ..	9·15–11·7	10·3	8·95–11·5	9·8

Lead in blood and tissues. The values recorded in Table 2 show that the lead content of the blood and tissues of affected lambs varied over a wide range and that in many instances, but not in all, it was abnormally high. The maxima of the normal ranges quoted in this table

were deduced from a consideration of our own values together with those published by Allcroft (1950) for sheep with no unusual exposure to lead. Such limits must be greatly exceeded before toxic effects are apparent, but with the data available at the present time it is not possible to define the lower limits of the pathological range with any certainty. Seven of the 25 cases examined showed normal blood values and in 5 of these the lead content of the rib was also within normal limits. All the values for liver and kidney lie above these limits but some do not greatly exceed them. For instance 4 values for liver and 7 for kidney lie between 10 and 20 p.p.m. D.W. On the other hand 16 cases had liver values of over 40 p.p.m. which are comparable with those found in calves which have died of acute lead poisoning (Allcroft and Blaxter, 1950). These were associated with correspondingly high values in the blood, kidney and rib.

TABLE 2

LEAD CONTENT OF BLOOD AND TISSUES OF AFFECTED AND NORMAL LAMBS

Tissue	*Affected lambs*			*Approximate maximum of normal range*
	No.	*Range*	*Mean*	
Blood, μg. Pb/100 ml. ..	25	2·9–250	70·3	25
Liver, p.p.m. Pb D.W.	28	10·4–219	58·2	10
Kidney, „ „ „	25	11·4–506	77·0	10
5th Rib, „ „ „	27	13·1–754	275	20
Lumbar Vertebrae, „ „ „	3	248–401	314	20

Over 90 p.p.m. of lead were present in the kidneys of 6 cases and in 5 of these there was evidence of damage to this tissue which is known to be caused by lead viz., the presence of acidophilic intranuclear inclusion bodies which were described above. In 3 of these lambs similar bodies were observed in the liver and were again associated with very high lead values (49·2, 94·5 and 219 p.p.m. D.W.).

Lead in pasture and soil. The lead content of 7 herbage samples collected from 2 affected areas near the old smelting mills in June while the disease was still occurring varied from 162 to 764 p.p.m. D.W. about a mean value of 278 p.p.m. Samples collected at the same time from pastures in the vicinity on which the disease does not occur contained 60·4 and 45·3 p.p.m. lead. In districts where lead does not occur geologically these values rarely exceed 10·0 p.p.m. The highest value was for a sample collected on the banks of a stream on Farm B which had been contaminated by refuse washed down from the smelting mill and the ore washing tanks, and there was evidence that this process was still going on. The farmer had

always associated this area with a particularly high incidence of the disease, and at one time it had been fenced off to prevent the sheep from gaining access to it. Samples of the soil taken here contained 19,500 and 17,800 p.p.m. lead D.W. which were soluble in 0·5N acetic acid while a sample from an unaffected pasture in the vicinity contained 14·6 p.p.m. In areas where lead does not occur geologically this value is generally below 5·0 p.p.m. While this survey was of a preliminary nature it does show that on at least 2 farms the amount of lead ingested by the sheep must be exceedingly high.

Zinc in blood and tissues. The results summarised in Table 3 indicate that the affected lambs contained unusually large amounts of zinc. Those for blood and rib show a statistically significant difference between affected and normal lambs ($P = <·01$ and $·02—·01$ respectively), while two thirds of the liver values for affected cases are greater than the highest figure obtained for a normal lamb. Comparison of Tables 2 and 3 shows that zinc is not deposited in the tissues from the blood stream to the same extent as lead and that it has relatively little affinity for the skeleton.

TABLE 3

ZINC CONTENT OF BLOOD AND TISSUES OF AFFECTED AND NORMAL LAMBS

Tissue	*Affected lambs*			*Normal lambs*		
	No.	*Range*	*Mean*	*No.*	*Range*	*Mean*
Blood, μg. Zn/100 ml.	16	279–531	369	6	154–258	203
Liver, p.p.m. Zn D.W.	9	85–430	172	6	62·2–118	92·8
5th Rib, „ „ „	20	82·1–186	114	6	74·8–99·5	84·5

DISCUSSION

The histological studies described above showed clearly that in all of the 20 cases examined in detail there was a generalised deficiency of osteoid tissue and that this was the only abnormality which was common to all. This diminution in bony mass was also demonstrated by density determinations on tibias from 6 of the most severely affected cases which had been in cold storage for about a year. Values ranging from 0·530 to 0·833 g. per ml. with a mean of 0·727 were obtained compared with 0·918 to 1·08 (mean 1·01) for tibias from 3 normal lambs of the same age which had been stored with them.

A conventional mineral analysis of dry fat-free ribs from the 20 cases gave normal results, showing that all the osteoid tissue which had been formed was calcified in the normal manner, and the analysis of blood samples demonstrated that there was no deficiency in the amounts of calcium and phosphorus in circulation. This condition

is thus readily distinguishable from rickets and it is obvious that it is caused by a failure in the synthesis rather than in the calcification of osteoid tissue.

In all except 4 of the 32 cases which have been examined so far the paralysis, lameness or stiffness was caused by lesions which resulted from a mechanical weakening of the bones by osteoporosis viz., spinal lesions, fractures and deformities in the long bones. Three of the 4 exceptions were affected by joint-ill and their lameness was due to polyarthritis but histologically they too showed osteoporosis. The other lamb was also osteoporotic but no lesions were found to account for its stiff gait. The spinal lesion which always occurred in the lumbar vertebrae was the most striking manifestation of osteoporosis and has not apparently been previously recorded in sheep or any other farm animals. It was present in half the lambs examined and has now been seen in lambs from 4 of the 7 farms known to be affected by the disease. This lesion was frequently accompanied by herniation of the nuclei of the intervertebral discs through the end plates of the vertebral bodies. It is interesting to note that in human osteoporotic conditions the changes are most severe in the spine, particularly in the lumbar region where according to Stein, Stein and Beller (1955) there is usually either compression of the vertebrae or herniation of the nucleus pulposus.

The study of osteodystrophic disease of the sheep is one which has received comparatively little attention in this country, particularly with regard to histopathology. Biochemical and pathological examinations are an essential and inseparable combination in the investigation of such conditions, since incomplete and possibly even false impressions of their nature may be obtained where only one of these methods is employed.

In this country two osteoporotic conditions have been described in ruminants hitherto viz., cappi or double scaup in hoggs in Northumberland by Bosworth and Stewart (1933), and cripples in young lambs in the North of England by Stewart (1933) and Piercy (1934), but the aetiology of both is still obscure. The disease we have described above shows many similarities to these conditions, the frequent occurrence of the characteristic lesion of cappi being particularly striking. Diseases of lambs which show clinical similarities to that described in this paper have been reported in other lead mining areas in the British Isles, but no histopathological studies have been described. Morgan (1924) mentioned that posterior paralysis occurred in lambs grazing in the vicinity of the lead mines in Cardiganshire, and Gardner (1924) reported that lambs in similar districts in the North of England were born with "rickety" backs. Recently Stewart and Allcroft (1956) described a condition of lameness and unthriftiness which occurs in the lead mining districts of Cumberland and Yorkshire. The lameness, which sometimes progresses to paralysis, is associated with sluggish flexor and extensor reactions and an under flexing of the hocks and fetlocks.

There is no suggestion of a disease process involving the skeleton. Unusually high levels of lead were found in the pasture and in the tissues of affected lambs which are of the same order as those found in many of our cases (Table 4).

Swayback was very prevalent in the lead mining districts of Derbyshire before the prophylactic value of copper supplements was discovered, and the high lead content of the tissues of affected lambs suggested to early workers that this element might play some part in the actiology of the disease (Innes and Shearer, 1940; Shearer, Innes and McDougall, 1940; Shearer and McDougall, 1944). It can be seen from Table 4 that these values are on the whole much lower than those found in the present investigation. Osteoporosis does not appear to be a feature of swayback, but on clinical evidence alone swayback might easily be confused with the condition we have described. Similarly without pathological examination it might be difficult to distinguish the latter from paralysis due to a spinal abscess (Marsh, 1932), or from muscular dystrophy due to Vitamin E deficiency.

The amount of osteoid tissue produced in the skeleton depends on the rate of production of osteoblasts, their activity and the availability of metabolites necessary for the synthesis of collagen and the cement or ground substance. In the disease we have described the osteoblasts are sometimes reduced in number and their production of osteoid tissue is considerably depressed. The close association of the disease with pastures contaminated by lead mining and smelting suggests that it is caused by some toxic substance, originally present in the mineral deposits. This substance could inhibit the metabolic activity of the osteoblasts either directly or indirectly by making some essential metabolite unavailable to them. Lead with its high affinity for the skeleton is obviously the first element which should be considered as a possible actiological agent. Although it was found that the lead content of the pasture and the blood and tissues of many affected lambs was unusually high and that the higher tissue levels were associated with tissue damage known to be caused by lead viz., basophilic stippling of the red cells and the presence of inclusion bodies in the liver and kidneys, the other lambs showed no tissue changes which could be attributed to this metal and the lead levels in some of these cases were within the normal range or only slightly elevated above it. Other features of chronic lead poisoning such as anaemia, encephalopathy and a blue line in the gums were entirely absent. Furthermore lead has never been known to produce osteoporosis and there are very few reports in the literature of skeletal changes of any kind associated with lead poisoning. Finner and Calvery (1939) mentioned that the bones of rats and dogs which had been poisoned with lead acetate appeared to be very fragile but did not describe their histological appearance. There have recently been reports of abnormalities in the bones of children suffering from lead poisoning, particularly in the metaphyseal ends of the long bones

TABLE 4

LEAD LEVELS ASSOCIATED WITH LOCOMOTOR DISABILITY IN LAMBS

Author	*Condition*	*Pasture* p.p.m. D.W.	*Blood* µg/100ml.	*Liver* p.p.m. D.W.	*Kidney* p.p.m. D.W.	*Rib* p.p.m. D.W.
Innes and Shearer (1940)	Swayback	41·1–258 (158)	—	2·5–25·1 (8·6)	—	—
Shearer, Innes and McDougall (1940) ..	Swayback	37–215 (96·5)	(24)	(12)	—	(15·6)*
Shearer and McDougall (1944)	Swayback	6·0–91·1 (20·4)	17 38 (24)	5–27 (13)	—	9·5 37·5† (23)
Allcroft (1950)	Swayback	—	—	14·8–30·4*	5·0–15·0*	—
Stewart and Allcroft (1956)	Locomotor Disability	(427)	54 270 (140)	44–376* (121)	27 715* (192)	—
Butler, Nisbet and Robertson (this paper) ..	Osteoporosis	162–764 (278)	2·9–250 (70·3)	10·4 219 (58·2)	11·4–506 (77·0)	13·1–754 (275)

* Calculated from the original results assuming that the water content of the fresh liver, kidney and rib was 75, 80 and 25% respectively.

† Calculated from the original results assuming that the ash content of the dry ribs was 50%.

The figures given in brackets are mean values.

(Follis, 1948; Anderson, 1953; Marsden and Wilson, 1955; Travers, Rendle-Short and Harvey, 1956), but these cannot be classed as osteoporotic changes. On the contrary the trabeculae are normally arranged and are increased in number and size, becoming closely packed and causing the characteristic dense zone on the X-ray plate. There was no histological or radiological evidence of changes of this kind in any of the lambs we examined, and there was no correlation between the severity of the osteoporosis and the lead content of the ribs. While most of the available evidence weighs against the incrimination of lead as the causative agent, it should be emphasised that no detailed study of chronic lead poisoning in lambs has so far been recorded.

It is certainly quite possible that some other substance is responsible for the disease since the ores of many other metals occur in the area. Minerals of 18 different metals have been identified and many of these contain minor quantities of a wide range of other elements (Temple, 1954, 1956). Zinc seemed to merit early investigation since its sulphide, sphalerite, occurs in workable quantities and it had been reported to cause unthriftiness and lameness in pigs (Grimmett, *et al.*, 1937; Sampson, *et al.*, 1942). The values for the zinc content of tissues of affected lambs recorded in Table 3 suggest that the amount of zinc absorbed by these animals was unusually high, but they are much lower than those which have been found in other animals suffering from zinc poisoning. For instance the values reported by Grimmett *et al.*, (1937) and Sampson *et al.* (1942) for the livers of their experimental pigs are about ten times greater, and Harrison and Staples (1955) concluded that values which are three to five times greater are of little toxicological significance in calves. The lameness in pigs suffering from zinc poisoning is caused by arthritis, but no detailed histological examination of the skeleton has been reported. The description of the gross findings of Sampson and his co-workers suggests that changes had also taken place in the bones.

The possibility that an excess of some element such as zinc might modify the toxic action of lead should be borne in mind. On theoretical grounds this is certainly feasible it if is assumed that metals exert their toxic effects by combining with complex-forming substances, such as amino acids, purines and pterins, so inhibiting the normal metabolism of these substances. The production of these biochemical lesions may often involve the displacement of essential metals from their natural combination with these compounds, thereby inhibiting the physiological function of these elements as well. It has been shown that lead and zinc have a similar affinity for such substances (Monk, 1951; Perkins, 1952; Butler, 1952; Tanford, 1952; Klotz, Urquhart and Fiess, 1952), and they will therefore compete with each other for them.

Vitamin C is an example of a metabolite required for osteoblastic activity which forms complexes with metals, such as lead (Kety, 1942) and copper (Olson and Brown, 1944), and may in consequence be

susceptible to inactivation by a toxic metal. Evidence that an interaction of this kind does take place in the body with lead, for instance, may be provided by reports that this vitamin has both a therapeutic and a prophylactic value in lead poisoning (Holmes, Campbell and Amberg, 1939; Pillemer, Seifter, Kuehn and Ecker, 1940; Marchmont-Robinson, 1941). Both the gross and histological characteristics of the osteoporotic condition produced by a mild deficiency of vitamin C in children (Eddy and Dalldorf, 1938) and guinea-pigs (Ham and Elliott, 1938) are very similar to those of the disease we have described in lambs. It is of course generally accepted that ruminants are not dependent upon dietary supplies of Vitamin C since they are able to synthesise it for themselves, but there is some evidence that this synthesis does not begin until they are 2 to 3 weeks old, and that at this stage they may be dependent to some extent on dietary supplies for the maintenance of normal health (Lundquist and Phillips, 1943; Cole, Rasmussen and Thorp, 1944).

Copper is also essential for osteoblastic function, possibly in association with Vitamin C. Osteoporosis accompanied by fractures has been reported in lambs and young cattle suffering from simple or conditioned copper deficiency in New Zealand (Cunningham, 1950). In the latter case the deficiency was induced by an excessive intake of molybdenum. It has also been shown that osteoporosis is one of the principal features of simple copper deficiency induced experimentally in puppies (Baxter and Van Wyk, 1953; Baxter, van Wyk and Follis, 1953) and pigs (Follis, Bush, Cartwright and Wintrobe, 1955). The disease we have described is fundamentally similar to these conditions, and in this connection it should be mentioned that there is a local belief that the incidence is reduced if the ewes are provided with copper licks. However we have not yet had an opportunity to assess the value of such measures.

The possibility that the disease is caused by the secondary effect of a toxic substance should also be considered. Kidney lesions were found in the majority of the affected lambs, but it was felt that they were too slight in degree and extent to cause a serious loss of some nutrient such as protein which is required for osteoblastic activity. It is possible that they were produced by an excessive urinary excretion of calcium, which has been shown to occur in the early stages of osteoporosis and to produce kidney damage (Albright, Burnett, Cope and Parson, 1941; Reifenstein and Albright, 1944). Further studies of the disease are in progress and will be described in a later communication.

CONCLUSIONS

An osteoporotic condition of young lambs which has occurred regularly for many years in an old lead mining area in Southern Scotland is described.

The clinical features are unthriftiness, fragile bones, a stiff gait

or lameness, and posterior paralysis. The latter is invariably caused by a lesion in one or more of the lumbar vertebrae which compresses the spinal cord. This also occurs in many of the less severely affected lambs and is the most striking manifestation of a generalised osteoporosis. Lameness may also be caused by fractures and deformities in the long bones. The frequent occurrence of the characteristic lesion of cappi in affected lambs and in the hoggs in these flocks is noted. Mineral analysis of the blood and bone of affected lambs gave normal results.

The lead content of the soil and pasture and the blood, liver, kidneys and bones of many affected lambs is unusually high, but the condition does not present a classical pathological picture of chronic lead poisoning. In those lambs with the higher lead values there were signs of tissue damage known to be caused by lead, viz. basophilic stippling of the red cells and the presence of acidophilic inclusion bodies in the liver and kidney, but these were the only recognisable features of lead poisoning present.

Unusually high values were also obtained for the zinc content of the blood and tissues of affected lambs. The possible involvement of these elements and chelating metabolites such as Vitamin C in the aetiology of the disease is discussed.

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LEGENDS TO ILLUSTRATIONS

Fig. 1. Posterior paralysis caused by collapse of a lumbar vertebra.

Figs. 2 and 3. Limb deformities produced by multiple fractures.

Fig. 4 above. Median vertical section of lumbar region of spine with cord in situ. The third lumbar vertebra has collapsed causing marked compression of the cord. A similar but smaller lesion is also present in the sixth vertebra. Herniation of the nucleus pulposus of three intervertebral discs can be seen.

Fig. 4 below. Similar specimen. The fifth lumbar vertebra has collapsed, the dorsal surface projecting into the spinal canal. Herniation of the nuclei of three intervertebral discs has occurred.

Fig. 5. Median vertical section through a typical collapsed lumbar vertebra showing compression and marked projection on dorsal surface, fractures of both epiphyseal cartilages and herniation of the nuclei of the adjacent intervertebral discs. H and E × 3.

Fig. 6. Vertical section of proximal extremity of affected tibia (left) showing marrow extending to epiphyseal cartilage and deficiency of cancellous bone in epiphysis and metaphysis. Normal tibia (right) for comparison.

Fig. 7. Cartilage-shaft junction at proximal extremity of humerus. Gross deficiency of trabecular bone can be seen. H and E × 32.

Fig. 8. Similar section from normal humerus for comparison with Fig. 7. H and E × 32.

Fig. 9. Vertical section of proximal extremity of affected tibia illustrating marked deficiency of cancellous bone. Note fracture of cortical bone of shaft at bottom left, and displacement of the abnormally extensive marrow. H and E × 2.

Fig. 10. Similar section of normal lamb tibia for comparison with Fig. 9. H and E × 2.

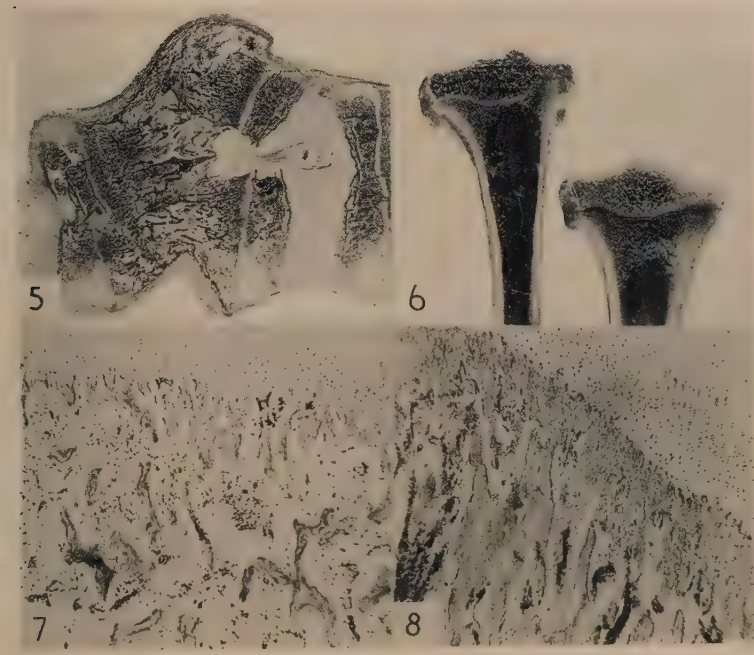
Fig. 11. Transverse vertical section through frontal bone and frontal sinus of skull of affected lamb. Marked thinning of the bone of the outer plate and of the septum is seen. H and E × 3.

Fig. 12. Similar section of normal lamb skull for comparison with Fig. 11. H and E × 3.

Fig. 13. Costochondral junctions of affected lamb (left) and normal lamb (right). H and E × 3.

Fig. 14. Longitudinal sections of rib-shafts from affected lamb (left) showing severe osteoporotic change, and from normal lamb (right). H and E × 3.

Fig. 15. Section of kidney cortex from osteoporotic lamb. Three glomeruli show swelling of the epithelial cells of Bowman's capsule, atrophy of the glomerular tuft and periglomerular fibrosis. H and E × 70.



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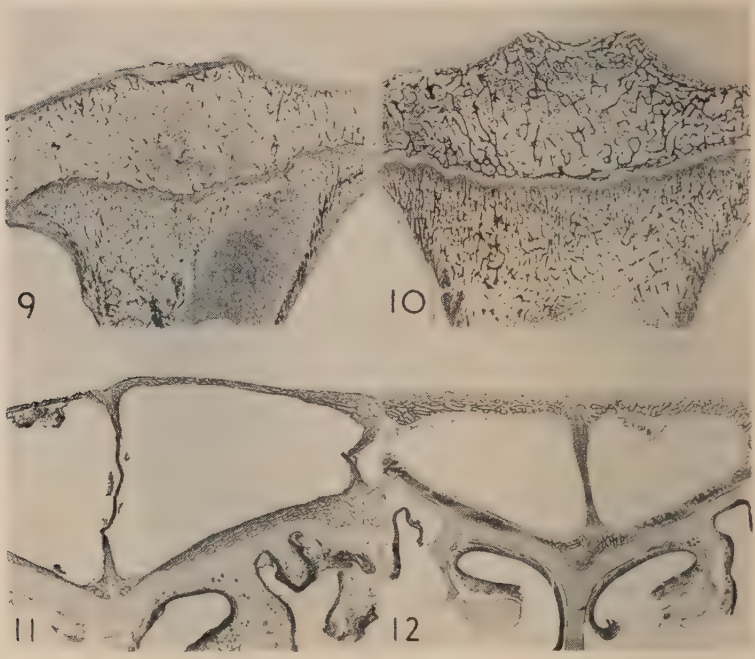
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A HISTOLOGICAL STUDY OF THE SWEAT GLANDS OF NORMAL AND DRY-COATED HORSES

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INTRODUCTION

This study was made as part of an investigation into the condition of "dry-coat" in horses. Dry-coat is characterised by the loss of the ability to sweat in horses that have been imported from more temperate and less humid climates to hot humid climates such as exist in coastal areas in Malaya and India.

It has been shown that changes in certain physiological factors take place in the condition (Lovatt Evans, Ross, Smith and Weil-Malherbe, 1957) but until now no comprehensive study of the histology of the skins of dry-coated animals has been undertaken. Such studies as have been made show conflicting results. Wright and Tull (1925) and Stewart (1938) reported that the sweat glands were atrophied, but Barnes (1938) found no change in them.

No study of the structure and histochemistry of the sweat glands of normal horses has been made in recent years. Gurlt (1835) examined the skin from various body regions of horses and reported that the sweat glands in the skin of the external genitalia were large and coiled with their ducts opening into the hair follicle orifices, while the sweat glands of the rest of the body surface were smaller and coiled with their ducts opening freely on the skin surface. However, Stoss (1906) found that all the sweat glands were associated with hair follicles. Therefore, in accordance with Schiefferdecker's (1922) classification, they have been looked upon as apocrine sweat glands. Another characteristic of apocrine glands is their mode of secretion, viz., a necrobiotic discharge of secretory cytoplasm. According to Trautmann and Fiebiger (1949) the sweat glands of horses secrete in that manner. A further point of differentiation between apocrine and eccrine glands has been found. Bunting, Wislocki and Dempsey (1948) have shown that glycogen is present in the secretory cells of human eccrine sweat glands but not in those of human apocrine sweat glands. This finding has been verified by other workers (Montagna, Chase and Lobitz, 1952, 1953a, b). The secretion of the apocrine sweat glands of the human axilla has been described by Shelley and Hurley (1953) as a translucent, sometimes cream-like fluid which appears in tiny droplets, never in large quantities, at the opening of the hair follicle orifices. This is in contrast to the copious secretion of the sweat glands of normal horses. In their discussion Shelley and Hurley mentioned that if the apocrine gland were to produce large quantities of clear sweat one

might expect to find glycogen within the secretory cells. Therefore as the sweat glands of the horse are apocrine glands which produce large quantities of sweat it was decided to study their histochemistry to see if their secretory cells contained glycogen and also to determine whether they contained lipids, iron, and hexosamine-containing compounds, all of which have been demonstrated in human apocrine sweat glands (Bunting *et al.*, 1948; Montagna *et al.*, 1953b). Shelley, Horvath, Weidman and Pillsbury (1948) have shown that sweat retention (anhidrosis) can be produced experimentally in man by anodic iontophoresis of the skin with saline or water. Shelley and Hurley (1953) have found that the apocrine glands of the human axilla show a refractory period of several hours after both normal secretory activity and drug stimulation. It is thought that this refractory period is the time required for regeneration of the apocrine gland epithelium.

As it seems likely that the condition of dry-coat might be connected with either of these experimentally produced anhidrotic conditions, two of the above methods, iontophoresis of saline and intradermal adrenaline injection, were carried out on horses, and the anhidrotic areas (not responding to intradermal adrenaline injection) were compared with corresponding normal areas from the same animals and with anhidrotic skins from Malaya.

MATERIALS AND METHODS

Normal horses. Skin samples from the neck, tail, perineum and muzzle of two normal horses and from the neck only of a third horse were obtained at autopsy at Porton.

Experimental horses. Skin samples from areas of the neck, rendered anhidrotic either by repeated intradermal injections of adrenalin or by anodic iontophoresis with saline (1 ma./cm^2 for 10 min. on two successive days), along with samples from control areas were again obtained at autopsy at Porton.

Anhidrotic horses. Two pieces of skin from the neck of a chronic dry-coated horse, Young Clem, were obtained at autopsy at Ipoh, Malaya. Small biopsy samples of skin were taken from the neck, rib or gluteal region of twenty-five dry-coated horses at Penang, using the method and rotary punch recommended by Urbach and Shelley (1951). The advantages of this method are the elimination of the use of a local anaesthetic when taking skin from the live animal and the rapid healing of the very neat, inconspicuous wound which results.

The apparatus required consists of an ordinary electric hand drill capable of 2,000 r.p.m., two small, hollow-ended steel bits measuring $3\frac{1}{2}$ cm. long with diameters of 2 mm. and 4 mm. at their razor-sharp cutting ends and two perspex plates, 25 x 25 x 3 mm., perforated centrally, one to fit each bit. The bits and the plates can be seen in Fig. 1.

The bit required is inserted into the chuck of the drill, care being taken to see that it is securely and centrally fixed in order to eliminate any eccentric movement. The perspex plate is placed firmly against the skin over the selected site which should be devoid of obvious subcutaneous veins. The bit is inserted into the hole in the plate (Fig. 2). The plate has two functions. It guides the cutting tool at right angles to the skin surface and, by preventing the chuck from reaching the skin surface,

does not allow the bit to penetrate too deeply and so damage the subcutaneous tissue. When the animal is standing quietly the drill is switched on and once maximum speed is attained the bit is allowed to touch the skin. A momentary touch is all that is required and no pressure other than the weight of the drill is necessary. The separated piece of skin remains *in situ* and can be lifted out with a pair of forceps and, if necessary, freed by scissors from any subcutaneous attachment (Fig. 3).

If any excessive pressure is applied or if there is any eccentric motion of the bit, it is found that the small piece of skin is very often lost through having been thrown aside by the centrifugal action of the drill. It was not necessary to apply any form of restraint to the animals as the effect of biopsy sampling in this manner was comparable to that of the use of a large bore hypodermic needle. It was advisable to avoid switching on and off the electric motor prior to collection as the noise was found to be disturbing to some animals.

The samples were fixed in either 6 per cent formol saline for routine examination or in an ice-cold mixture of 9 parts saturated picric alcohol plus 1 part neutral formalin for the detection of glycogen. It was subsequently found that the latter fixative made the skin extremely hard and difficult to section, but that after fixation in the formol saline glycogen could still be demonstrated without this difficulty occurring. Some of the material from normal and experimental horses was sectioned on the freezing microtome at 15μ previous to staining for the detection of lipids. The rest of the material was embedded in paraffin and subsequently sectioned at 8μ.

Paraffin sections were stained with Delafield's haematoxylin and eosin to examine the general structure. Both Best's carmine and the periodic acid-Schiff (P.A.S.) test of Hotchkiss (1948) were used on paraffin sections to test for glycogen. Control sections were stained by both methods after treatment with salivary diastase to remove glycogen. A positive P.A.S. reaction after diastase indicates the presence of hexosamine-containing compounds such as mucoproteins and mucopolysaccharides. To differentiate between these various hexosamine-containing compounds sections were stained in 1 per cent toluidine blue for 10 min. and were examined for metachromasia in water and in glycerine jelly. Paraffin sections of normal skin only were stained by Perl's method for ferric iron and by the Turnbull blue method for ferrous iron.

Frozen sections of normal skin were stained in Sudan black B in propylene glycol (Chiffelle and Putt, 1951) to test for lipids. Control sections were passed through absolute alcohol, acetone and chloroform before staining. Frozen sections were also tested for cholesterol and cholesterol esters by Romieu's modification (1925) of the Schultz method.

RESULTS

Normal Horses

The sweat glands are tubular glands. Their secretory portions are in some cases coiled into balls which lie in the dermis at about the same level as the bases of the hair follicles. In other cases they are sinuous and lie alongside the hair follicles from the base of the follicle to the sebaceous gland level (Fig. 4). The excretory duct is a narrow straight unbranched tube which opens into the hair follicle orifice close to the skin surface. The secretory tubules con-

sist of two layers of cells, an inner layer of cubical secretory epithelium and an outer layer of myoepithelium. The cytoplasm of the secretory cells is clear. The nuclei are almost spherical and are situated centrally in the cells. In some of the tubules protuberances of the cytoplasm into the lumina can be seen. These protuberances are more opaque than the rest of the cytoplasm. No evidence of necrobiotic discharge was seen in any of the specimens examined. In most glands some granules of yellowish brown pigment occur in the secretory epithelium. A gland in normal horse skin can be seen in Fig. 5. The glands in the skin from the perineum are larger than those in the skin of the neck, tail or the muzzle. They also contain much larger amounts of pigment than do the glands in the other areas. In one of the blocks of skin from the muzzle there were no sweat glands present although there were hairs which had large multi-lobed sebaceous glands associated with them.

Granules of glycogen are present in the cytoplasm of the secretory cells. Sections stained with P.A.S. after treatment with diastase still showed a positive reaction of non-granular material in the secretory cells and in any secretion present in the lumina of the glands. Staining with toluidine blue showed metachromasia in the hair papillae and around the hair follicles, and faint metachromasia was present in the sweat gland basal membranes. However there was no metachromasia in the sweat gland tubules or secretion. This means that the P.A.S. positive material is either a mucoprotein or a mucopolysaccharide which is not strongly acidic.

Both Perl's and the Turnbull blue reaction gave negative results indicating that no inorganic iron is present in the sweat glands of the horse.

Staining with Sudan Black B showed very slight diffuse sudanophilia in the glands. The pigment granules were also slightly sudanophilic. The sudanophilia in the glands was still present in sections passed through alcohol, acetone and chloroform before staining. Sections stained by the Schultz test showed that there is no cholesterol in the sweat glands of the horse but that it is present in the sebaceous glands where various sized droplets of sudanophilic material which were slightly soluble in alcohol and completely soluble in acetone and chloroform, were detected.

Experimental Horses

The sweat glands in the areas of skin rendered anhidrotic by repeated intradermal adrenaline injections showed changes in their secretory epithelium. In the first animal the epithelium had undergone great damage. Fig. 6 is a photomicrograph of a typical sweat gland. The secretory cells were vacuolated and, in some places, completely ruptured. Some of the nuclei were fragmented. All were distorted and shrunken. Glycogen was not present in the secretory cells, although the sweat glands in the control areas, which structurally were normal, contained glycogen. In the second animal the

damage was not so severe. There was some vacuolation in a few gland tubules but the most common change that was present was a flattening of the secretory epithelium and of the nuclei. The gland tubules consequently had the false appearance of being distended. The chromatin appeared to be densely packed in the nuclei. There was a trace of glycogen in the secretory cells. Again the glands in the control area were normal.

The sweat glands in the areas of skin rendered anhidrotic by iontophoresis appeared to be quite normal and glycogen occurred in their secretory cells. In the second horse the sweat duct orifices were blocked by plugs of keratin (Fig. 7). Unfortunately, in the first horse, the presence of inflammatory lesions in the epidermis, especially around the openings of the hair follicles, made it impossible to see whether any blocking of the ducts had taken place. It is thought that these lesions were the result of shaving the skin the day before and were not due to iontophoresis since they also occurred in the control areas. The sweat gland ducts and tubules appeared to be normal.

Anhidrotic Horses

The skins of three of these animals appeared to be normal. It was impossible to section the sample from a fourth animal. In the skins of the remaining twenty-two animals, however, various abnormalities were present. There was blocking of the ducts in up to 10 per cent of the sweat gland units. The most common cause of obstruction was a plug of keratin in the duct orifice, such as occurred in the area of skin made anhidrotic by iontophoresis. There was often very slight inflammation of the epidermis round these blocked ducts. Below the site of obstruction the duct was sometimes distended in either its epidermal or dermal portion. Again, slight inflammation occurred round the distended ducts and inflammatory cells could be seen in the process of migrating into the duct lumina. Occasionally further plugging of the duct was present. This was sometimes due to a cast of homogeneous material, probably sweat, which contained some phagocytic cells. In a few distended ducts casts of epithelial cells caused further blockage. These cells were either in the form of tight whorls (Fig. 8) or of loose clusters. The secretory tubules of these blocked gland units were usually severely distended. Their epithelium and nuclei were extremely flat (Fig. 9). The unblocked sweat gland units appeared to be normal (Fig. 10). In a few cases the secretory epithelium was a little less cubical than normal. All the glands contained glycogen. In one or two animals which had suffered from the condition of dry coat for a long period, e.g. Young Clem, a few sweat glands had atrophied. Their secretory tubules were shrunken and the epithelium completely flattened. They were usually surrounded by an area of fibrosis. In those animals there also was evidence of much hair shedding.

DISCUSSION

It seems unlikely that the blocking of no more than 10 per cent of the sweat gland units can be responsible for the condition of anhidrosis. This assumption is supported by the fact that in three of the anhidrotic skins no abnormalities could be detected. The blocked gland units cannot be responsible for the anhidrosis but their presence must aggravate the condition and may cause irritation in the skin.

It is thought, however, that the changes here may be the result of anhidrosis rather than its cause. Shelley and Horvath (1950) have shown that maceration of the skin surface can bring about keratinous plugging of eccrine sweat ducts in man, and concluded that *miliaria rubra* (prickly heat) develops as a result of the macerating action which sweat, prevented from evaporating by a high humidity, has on the skin. Prior to the onset of anhidrosis many horses, during or following the period of acclimatisation, undergo a period of profuse sweating. Since it has been shown that the sweat glands of horses normally secrete in response to adrenaline (Lovatt Evans and Smith, 1956), it is thought that this profuse sweating is caused by an increase in circulating adrenaline which results from the subjection to heat stress (Lovatt Evans *et al.*, 1957); ultimately the sweat glands seem to become habituated to it and sweating ceases. However, it seems likely that during the period of profuse sweating maceration of the skin takes place with resultant plugging of some of the sweat pores. The distension of ducts and tubules probably results from this poral occlusion. It seems possible that plugging of the ducts prevents the normal proliferation of epithelial tissue and so leads to the abnormal growth of epithelial cells in the distended ducts.

In anhidrotic animals only slight sweating in certain body regions, viz., along the mane, round the throat and between the legs, takes place even after severe exercise or after intravenous or intradermal injections of amounts of adrenaline which would produce profuse sweating in normal animals. If the glands have in some way become immune to small increases in circulating adrenaline, this would explain why most of the glands appear, histologically, to be quite normal, though physiologically non-functional.

It has been mentioned that glycogen is present in the eccrine sweat glands of man but has never been found in human apocrine glands. The sweat glands of the horse are apocrine glands in so far as they are associated with hair follicles but, judging from their copious secretion, they behave more like eccrine glands. This is further supported by the fact that their secretory cells contain glycogen. Therefore it would seem that we are dealing with an intermediate apocrine-eccrine gland. It is probable that all skin glands are derived, phylogenetically, from the mucous glands of the surface of amphibians. The presence of mucin in the secretion

of the apocrine glands of man (Montagna *et al.*, 1953b) and of cattle (Lennox, Pearse and Symmers, 1952) indicates that they are primitive skin glands. It is thought that the eccrine sweat glands are the most highly evolved skin glands since they secrete large amounts of a fluid which contains no mucin. It seems reasonable, therefore, to assume that the sweat glands of the horse represent an intermediate step in this evolution. It is also apparent from these findings that Schiefferdecker's arbitrary classification of skin glands as apocrine or eccrine is not quite valid.

Yuyama (1935) and Shelley and Mescon (1952) have found that the only change which takes place in human eccrine glands during prolonged secretion is that glycogen disappears from their secretory epithelium. Thaysen and Schwartz (1955) have shown that the decrease in reflex sweating which takes place after prolonged exposure to a high environmental temperature is due to "fatigue" of the glandular unit and not to decreased nervous stimulation. It seems likely, therefore, that this "fatigue" is due to a depletion of the glycogen in the gland. A similar but more severe change seems to have taken place in the sweat glands in the area of skin made anhidrotic by repeated adrenaline injections in the first experimental horse. The glycogen has disappeared and furthermore the secretory cells are badly damaged. It is obvious that recovery from such damage will be a long process which will entail a replenishing of the secretory cells with glycogen. However, from the biopsy samples investigated, it can be seen that if such damage has occurred, the glands regenerate and glycogen deposits are built up in them once more. The fact that these glands cannot respond to increases in circulating adrenaline such as normally occur on exercise, nor to intradermally injected adrenaline, is explained by Lovatt Evans *et al.* (1957), as the result of accommodation consequent upon a sustained high level of adrenaline in the blood.

It is well-known that human eccrine sweat contains lactic acid, at a concentration 2 to 5 times that in plasma. Whitehouse (1935) showed that the concentration of lactic acid in human sweat is independent of its concentration in the blood, and Nitta (1942) found no difference in the lactic acid concentration between thermally-induced sweat and the sweat due to muscular exercise. Since it has been shown that during activity of the sweat gland glycogen is broken down, it may be assumed that the lactic acid in sweat is derived from the break-down of glycogen in the gland itself. It has also been observed that during prolonged sweating in man the lactic acid concentration of sweat decreases (Kuno, 1956). Since the glycogen in the sweat glands of horses undergoes the same changes as that in human eccrine glands it is to be expected that the sweat of horses contains lactic acid. In fact it does contain some though less than in plasma.

It was rather surprising to find that, in the sweat gland units of the second experimental horse which were blocked as a result of

iontophoresis, no distension of the sweat ducts or secretory tubules had taken place. However, Shelley (1952) has observed that in human skin the sweat retention vesicles which develop clinically in acute experiments and in *miliaria rubra* are not visible in histological preparations of these lesions. It was his opinion that dilatation of the duct must be both severe and prolonged for it to appear in histological preparations. Holyoke and Lobitz (1952) have noticed the same phenomenon and, from our findings, it appears that the same thing happens in horse skin. The blocking of ducts in the experimental animal was only of two days duration. In the anhidrotic animals, however, where the obstruction was longstanding, distensions of ducts and secretory tubules were demonstrable histologically.

CONCLUSIONS

The sweat glands of normal horses, like apocrine glands, are associated with hair follicles, but, like eccrine glands, they contain glycogen in their secretory cells and produce large amounts of a watery sweat. It is concluded that they represent an intermediate step in the evolution from the simple mucin-secreting apocrine gland to the highly-specialised eccrine gland.

In the skins of anhidrotic horses about 10 per cent of the sweat gland units are blocked by keratinous plugs but the rest of the glands seem to be normal. It is thought that the blocked glands are the consequence, rather than the cause of the condition of "dry-coat," which is the result of a physiological inhibition rather than of structural change.

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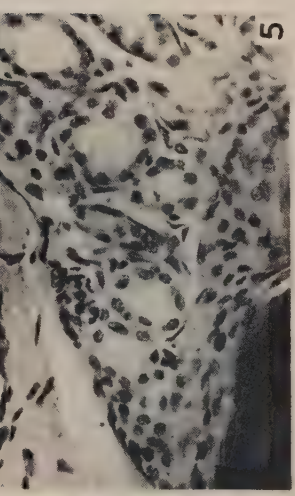
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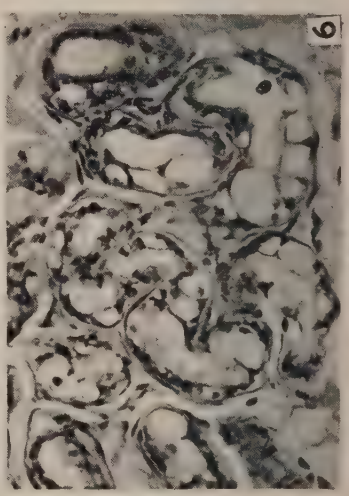
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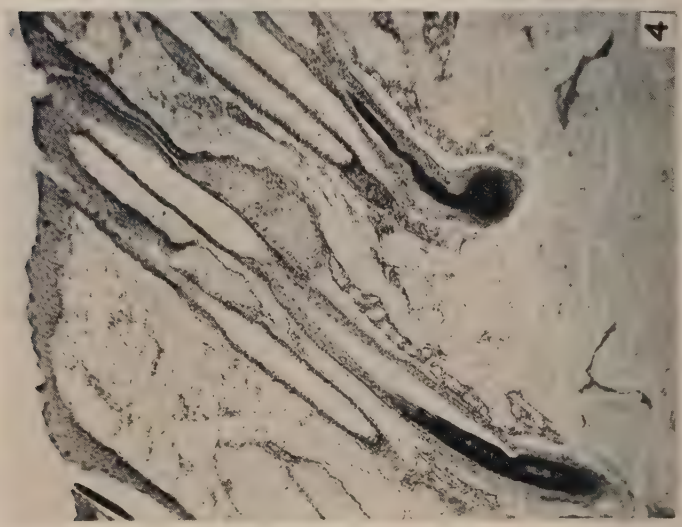
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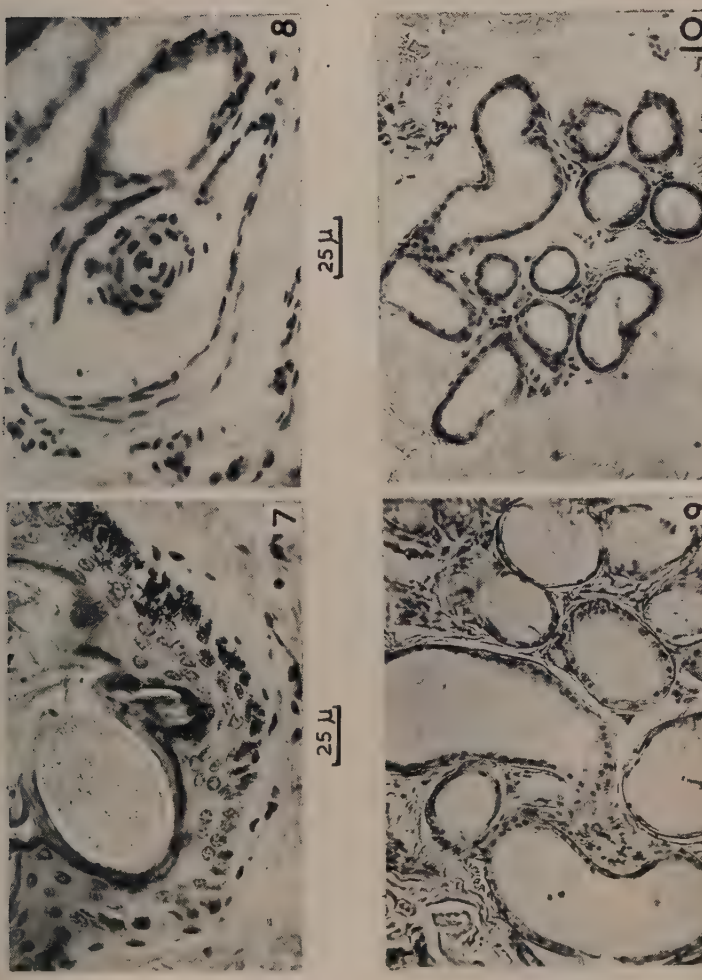
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LEGENDS TO ILLUSTRATIONS

Fig. 1. The steel bits used to take biopsy skin samples along with their respective perspex plates.

Fig. 2. Cutting a sample from the neck region.

Fig. 3. Removing the sample which was left *in situ* after being cut.

Fig. 4. A hair follicle unit in normal horse skin. The secretory portion of the sweat gland is tortuous and lies alongside the hair follicle. The excretory duct opens into the orifice of the hair follicle close to the skin surface.

Fig. 5. Secretory tubules of a sweat gland of a normal horse. The nuclei of the secretory cells are almost spherical and are situated centrally in the clear cytoplasm.

Fig. 6. Secretory tubules of a sweat gland in an area of skin made anhidrotic by repeated adrenaline injection. The secretory cells are vacuolated and sometimes ruptured. Their nuclei are shrunken and fragmented.

Fig. 7. The opening of a sweat duct into a hair follicle orifice in skin made anhidrotic by iontophoresis. The sweat duct is blocked by a plug of keratinous material.

Fig. 8. A distended sweat duct in a dry-coated horse. A cast of epithelial cells in the form of a whorl is present in the duct lumen.

Fig. 9. Distended secretory tubules in a blocked sweat gland unit in a dry-coated horse. The secretory cells have become flattened. In one or two places parts of their elongated myoepithelial cells can be seen.

Fig. 10. Normal sweat gland tubules in a dry coated horse.

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